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Effect of Salicylic Acid on Adrenal-Pituitary System. III. Studies on Mechanism of This Effect.* (21116)

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Salicylic acid (SAL) has in normal animals a specific effect on the adrenal-pituitary system which can be demonstrated by a depletion of adrenal ascorbic acid (AAA)(1-5) and adrenal cholesterol(3). Since the presence of an intact pituitary is necessary(3,4), it has been suggested that SAL stimulates directly or indirectly the production or release of increased amounts of ACTH. The present study was undertaken to investigate in some detail: 1. The duration of the SAL action; 2, The effect of repeated doses; 3. The effect of pretreatment with cortisone; 4. Attempts to determine the mechanism of action.

Methods and evaluation. The experiments were made in Wistar rats as described previously(4). All AAA values are given in per cent of that found in *untreated* controls. This eliminates daily variations(4). An injection of physiological saline solution produces a small, but significant depletion of AAA. In previous publications(4,5) saline controls were arbitrarily assigned a value of 100%.

TABLE I. Adrenal Ascorbic Acid of Rats, 2 Hours after Single Subcutaneous Injection of Saline Solution or Salicylic Acid.

	Adrenal ascorbic acid in % of	
	Saline controls (previous papers)	Untreated animals (present paper)
Untreated animals	135	100
Saline	100	75
SAL, 300 mg/kg	59	44

The present experiments can be described more clearly if AAA of untreated animals is considered to be 100%. Table I presents a comparison of the two forms of presentation.

Results. The results of time-effect experiments (Table II) permit 2 conclusions: 1. The maximum depletion of AAA required more than one hour. 2. The level of AAA had not returned to normal within 16 hours after 300 or 500 mg/kg of SAL. The SAL blood levels at the end of the 16-hour experiment were 6.7 and 11.2 mg %, respectively.

The question of adrenal response to repeated doses of SAL was investigated in 2 ways. First, it was necessary to establish the effect of a second dose of SAL given to animals in which the adrenals had not yet recovered

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TABLE II. Adrenal Ascorbic Acid of Rats after Single Subcutaneous Injection of Saline Solution or Salicylic Acid.

Time (hr)	1	2	3	16
	Adrenal ascorbic acid in % ± S.D. of untreated controls			
Saline	86 ± 9 (13)	75 ± 9* (87)	87 ± 11 (8)	97 ± 10 (20)
SAL, 300 mg/kg	52 ± 7† (10)	44 ± 6† (76)		74 ± 9† (8)
" , 500 "		38 ± 8† (6)		60 ± 10† (5)

No. in parentheses refers to No. of animals.
* Significantly lower ($p = 0.05$ or less) than untreated animals.
† Significantly lower than untreated animals and the corresponding saline controls.

completely from a first dose of the drug. Twelve animals were injected with 500 mg/kg each of SAL. After 16 hours, 6 of the animals were killed, while the other 6 received a second dose of SAL (300 mg/kg) and were sacrificed 2 hours later. AAA values in the 2 groups were 365 ± 27 mg % and 201 ± 9 mg %, respectively.‡ The responsiveness of the adrenals was not impaired by the first dose of SAL. The per cent depletion from the second dose was of almost the same magnitude as that found after a single injection of 300 mg/kg of SAL and greatly exceeded the effects of repeated saline injections (Table III).

The next step was an experiment with repeated doses extending over 96 hours. Three groups of 8 animals each received on 4 successive days 2 injections daily of either 10% SAL (200 mg/kg at 8 A.M. and 300 mg/kg at 4 P.M.) or saline solution (2 and 3 ml/kg, respectively), or a 4.23% solution of sodium chloride (2 and 3 ml/kg) which is equimolar to a 10% SAL solution. All animals were killed 16 hours after the last injection. Repeated injections of sodium chloride, either as isotonic or as hypertonic solution, did not deplete AAA. On the contrary, a slight, but significant increase was observed, suggesting overcompensation (rebound) due to the repeated, non-specific stress of sodium chloride injections. The effect of SAL was the same after the last as after the first injection (compare with Table II). In both cases, AAA had not returned to normal values within 16 hours. Thus, repeated doses of SAL did not exhaust the adrenals or impair their responsiveness, as indicated by AAA. The adrenal weights in the 3 groups were not different from each other or from that of untreated animals (30.0 ± 3.2 mg).

The effect of pretreatment with cortisone on the activity of SAL is summarized in Table IV. Cortisone acetate§ was injected intramuscularly in doses from 0.16 to 80.0 mg/kg, followed 16 hours later by a standard subcutaneous dose of SAL (300 mg/kg), or saline. The animals were killed 2 hours after the second injection. Under these conditions doses from 0.16 to 0.64 mg/kg of cortisone had no influence on the SAL action. In the range from 0.90 to approximately 8.0 mg/kg of cortisone, the effect of SAL was gradually reduced, while above 10 mg/kg even an 8-fold increase of cortisone had no measurable additional influence. The saline controls were affected in the same manner as the SAL animals, while cortisone alone did not produce changes in AAA. Although the experimental data do not exclude the possibility that still larger doses of cortisone would have a greater inhibitory effect, the observed dose-response relationship makes this rather unlikely. The results agree with observations by Hetzel and Hine(2) which, however, covered a much narrower dose range of cortisone.

All previously described experiments had extended the original observation that SAL acts on the adrenal-pituitary system by an increased production or release of ACTH. To obtain some information on the mechanism of this effect, animals under pentobarbital anesthesia were investigated. Pentobarbital sodium (PEN) in a dose of 40 mg/kg was injected intraperitoneally. As soon as the animals were in complete anesthesia (usually after 1-2 minutes), either SAL or physiologic saline was given subcutaneously. Duration

‡ This and other values with standard deviation.

§ Generously supplied by Merck and Co.

TABLE III. Adrenal Ascorbic Acid of Rats after 4 Days of Subcutaneous Injections of Salicylic Acid or Physiologic or Hypertonic Sodium Chloride Solution.

Drug	Daily dose	No. of animals	Adrenal wt in mg (± S.D.)	AAA in % of untreated animals (± S.D.)
SAL	AM: 200 mg/kg PM: 300 "	8	28.8 ± 3.8	82 ± 14†
Saline	AM: 2 ml/kg PM: 3 "	8	31.4 ± 3.5	110 ± 17
4.23% NaCl*	AM: 2 " PM: 3 "	8	30.9 ± 4.1	114 ± 8†

* Equimolar to a 10% solution of Na salicylate.
† Significant difference ($p = 0.05$ or less) from untreated controls.

of the experiments was 2 hours, following SAL injection (Table V). AAA was reduced to 82% by PEN alone and to 70% by PEN plus saline. When 300 mg/kg of SAL was administered to 16 anesthetized animals, AAA depletion reached 58%. However, the actual data centered around a low and a high value and this classification coincided exactly with gross observations on depth and length of anesthesia. All animals which had shown signs of incomplete anesthesia or had started to wake up before the end of the experiment, had low AAA (258 ± 47 mg %), while the opposite was the case with all animals which stayed completely anesthetized during the experiment (352 ± 61 mg %). Therefore, this group of 16 animals is divided into 2 subgroups as indicated in the table. When anesthesia with PEN was complete, SAL (300 mg/kg) did not cause a significant depletion of AAA when compared to the corresponding control (PEN plus saline). When anesthesia was incomplete, the effect of SAL was the same as in non-anesthetized animals. All 4 animals receiving PEN + 600 mg/kg of SAL had shown signs of incomplete anesthesia, and did not differ from non-anesthetized animals.

TABLE IV. Effect of Salicylic Acid on Adrenal Ascorbic Acid of Rats following Pretreatment with Cortisone Acetate.

No. of animals	Cortisone, mg/kg	2nd drug	AAA in % ± S.D. of untreated controls
2	80	Sal, 300 mg/kg	56 *
4	40	"	55 ± 8*
4	20	"	54 ± 10*
6	10	"	54 ± 10*
6	5	"	51 ± 7*
6	2.5	"	49 ± 6*
6	1.25	"	47 ± 10
4	.62	"	44 ± 8
4	.31	"	43 ± 9
4	.16	"	44 ± 10
†	—	"	44 ± 6
8	40	Saline	88 ± 12
12	—	"	76 ± 8
8	40	—	101 ± 12

* Significantly different ($p = 0.05$ or less) from effect of SAL without pretreatment with cortisone.
† Previous data(4).

Discussion. The previously established specific effect of SAL on the adrenal-pituitary system lasts much longer than the non-specific stress of a saline injection, and it can be maintained by repeated administration of the drug. If one accepts depletion of AAA as an index of circulating ACTH, and since SAL acts only in the presence of an intact pituitary, the experimental data imply that the SAL induced stimulation of the pituitary continues as long as a minimum concentration of SAL is present in the circulation. This distinguishes the specific action of SAL (and similarly acting drugs) from the non-specific, short-lasting effect of an injection of saline or other inert materials.

It has been postulated by Sayers(6) that an autoregulatory mechanism governs the secretion of ACTH by the pituitary and of corticoids by the adrenal cortex. According to this concept, an increase in circulating ACTH results in an increased secretion of corticoids which in turn suppress further secretion of ACTH by the anterior pituitary until equilibrium is reestablished. Thus, injections of cortisone should suppress the normal "endogenous" secretion of ACTH. The observed partial inhibition of AAA depletion

TABLE V. Effect of Pentobarbital Anaesthesia on Adrenal Ascorbic Acid Depletion by Salicylic Acid.

Drug administered			No. of animals	SAL blood level, mg %	Adrenal ascorbic acid, % ± S.D.	Comment
PEN, mg/kg	Saline, ml/kg	SAL, mg/kg				
40	—	—	14	—	82 ± 16*	
40	3	—	11	—	70 ± 7*	
40	—	300	16	41	58 ± 23†	All animals
40	—	300	10	39	64 ± 17*	Fully anaesth.
40	—	300	6	45	47 ± 18†	Not fully anaesth.
40	—	600	4		39 ± 12†	" " "

* Significantly lower ($p = 0.05$ or less) than untreated animals.
† " " than untreated animals and the corresponding controls without SAL.

by pretreatment with cortisone may possibly be an index of this endogenous ACTH. However, the direct stimulatory effect of SAL is independent of the amount of circulating cortisone. This leads to the suggestion that the response of the pituitary to circulating corticoids and circulating SAL is mediated through different mechanisms. This explains also why the response of the adrenal to repeated injections of SAL is practically the same as to one injection.

This observation differs from that of Sayers and Sayers(7) that ascorbic acid depletion from exposure to cold or injection of epinephrine or histamine may be completely blocked by pretreatment with adrenal cortical extract. This discrepancy again suggests a difference between the mechanism of action of SAL and certain other "non-specific" stress agents.

The results in PEN anesthetized animals are taken as an indication that SAL acts directly or indirectly on the hypothalamus which in turn relays a stimulatory effect to the anterior pituitary. The following observations are in support of this assumption:

1. PEN is known to act on the hypothalamus, blocking the temperature regulating center. The antipyretic action of SAL is assumed to be mediated through the hypothalamus(8). 2. Barbiturates do not interfere with the ability of injected ACTH to deplete AAA(9,10). Thus, the blockade of the SAL effect by PEN indicates that in this case the release of ACTH is inhibited in the completely anesthetized animals. 3. The place of primary action of SAL must be different from that of injected histamine or epinephrine since the adrenal response to these agents is not inhibited by PEN(9). 4. Rats under PEN anesthesia do not respond to the stress of low temperature (3°C for one hour) which ordinarily results in depletion of adrenal ascorbic acid (9). 5. Evidence for a direct hypothalamic influence on the anterior pituitary has been presented by Harris(11), Greer(12), and Porter(13). 6. Hume(14) has proposed a hormonal mechanism in the hypothalamus capable of stimulating an increased secretion of ACTH.

The experimental data obtained with SAL under varying conditions indicate certain differences from other stress-producing agents such as histamine or epinephrine. To the observations following premedication with cortisone or pentobarbital may be added that the effect of SAL is not inhibited by adrenergic blockade(15). It is suggested that the SAL effect is mediated through the hypothalamus and that it differs from that of epinephrine and histamine and all those stressing conditions which involve one of the last named agents.

The absence of hypertrophy of the adrenals is contrary to most observations on the effect of ACTH and other stress producing agents. However, similar negative results have been reported(16). It has also been shown that ascorbic acid in large doses will prevent adrenal weight increases due to exposure to cold(17). Since SAL is known to have considerable influence on ascorbic acid metabolism, the explanation for the anomalous adrenal weights may perhaps be found in a study of ascorbic acid mobilization and synthesis under the influence of SAL.

The observation that the SAL effect was blocked by PEN only in 10/16 animals may be explained by the central stimulating effect of salicylates. Such an effect has been reported in salicylate poisoning(8). It was also indirectly observed in the present experiments, in that 6 of the SAL treated animals, but none of 25 controls showed signs of incomplete or shortened anesthesia.

Summary. The previously established specific action of salicylic acid on the adrenal-pituitary system has been investigated as to duration, effect of repeated doses of salicylic acid, effect of pretreatment with cortisone and possible mechanism of action. The effect of a single injection of salicylic acid (300 mg/kg) lasted more than 16 hours, at which time still appreciable bloodlevels of salicylic acid were present. Repeated injections of salicylic acid did not impair the responsiveness of the adrenals. Pretreatment with cortisone reduced, but did not abolish the effect of salicylic acid. Complete anesthesia with pentobarbital blocked the effect of salicylic acid. The results are interpreted as an effect of salicylic acid on the hypothalamus with subsequent stimulation of the pituitary.

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Intracellular Multiplication of *Toxoplasma gondii* in Adult Mammalian Macrophages Cultivated *in vitro*.* (21117)

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Toxoplasma gondii, an obligate intracellular parasite, can easily be propagated in cultures of different types of tissues of either embryonic or adult origin and the parasites have been maintained in serial passages in tissue cultures without detectable loss of virulence for mice(1,2). The organisms invade and probably multiply in cells derived from all 3 embryonic layers and there is no evidence for any cellular specificity. In experimental toxoplasmosis, especially after intraperitoneal infection, the parasites are mainly located in macrophages. These cells can be maintained in tissue cultures and provide a system for *in vitro* study of intracellular parasites(3). After preliminary experiments had shown that Toxoplasma do multiply in macrophage cultures, the ability of Toxoplasma to multiply *in vitro* within macrophages derived from susceptible and resistant animals was studied.

Materials and methods. Animals. Swiss white mice (Schwentker strain) were raised and supplied by the Department of Bacteriology and Immunology, Harvard Medical School. Rats, guinea pigs and rabbits were obtained from outside sources. The animals

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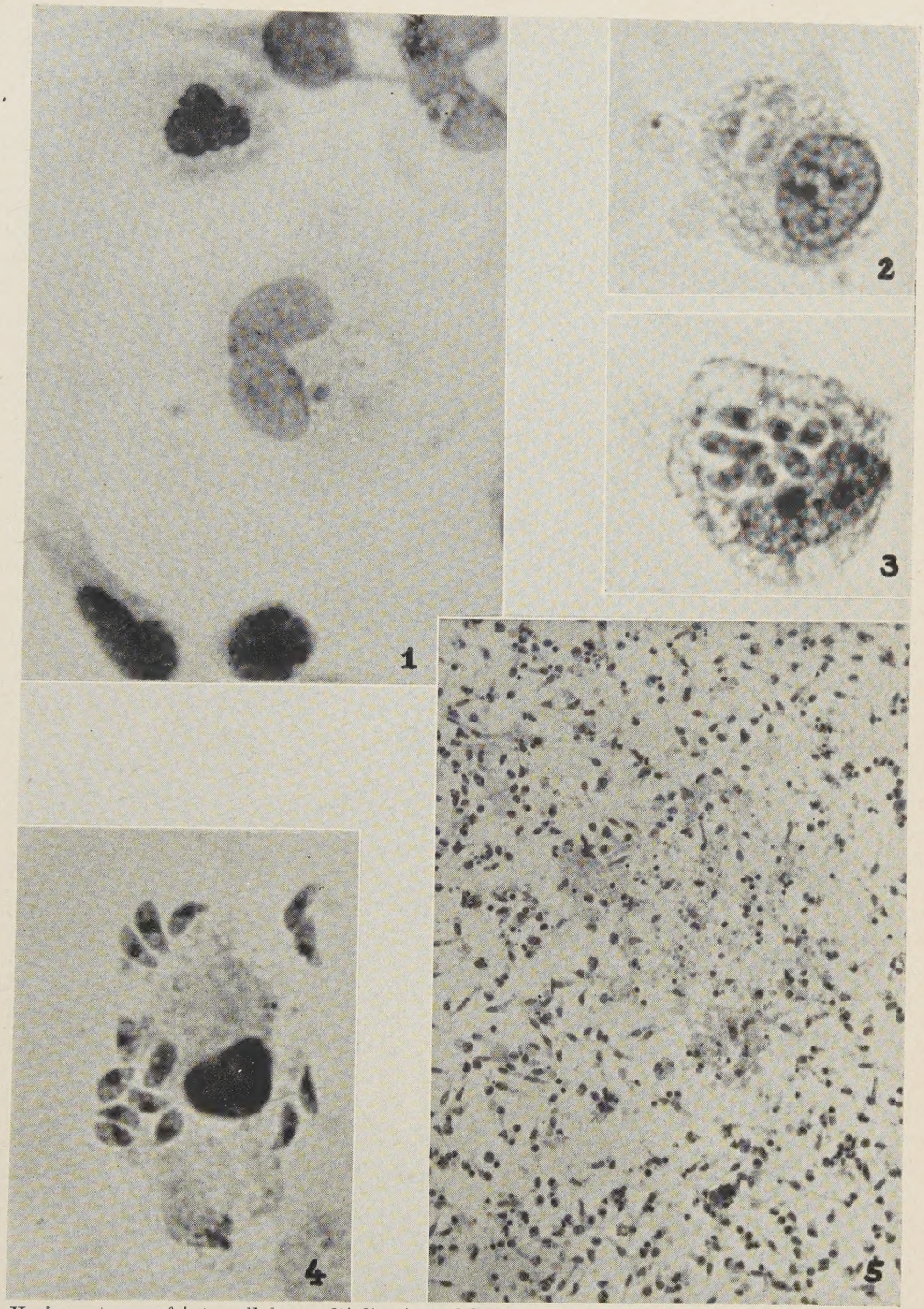
were fed with pellets and water, and the guinea pigs and rabbits supplied with cabbage *ad lib.*

Toxoplasma. The RH strain, originally isolated from a child by Sabin(4), was used throughout this study. It was obtained from 2 independent sources: one strain from Dr. Q. M. Geiman (Department of Tropical Health, Harvard School of Public Health, Boston), and the other through the courtesy of Dr. J. K. Frenkel (Department of Pathology and Oncology, University of Kansas, Kansas City.) The strains were maintained in mice. The content of the peritoneal cavity of a moribund mouse was collected and 0.2 ml of varying dilutions was injected intraperitoneally into fresh mice; 0.2 ml of a 10^{-7} dilution usually contained enough Toxoplasma to kill the mice within 7-14 days. To obtain a comparable infective dose of Toxoplasma for the infection of the macrophage cultures, mice were killed 4-5 days after infection with ether and were injected in the peritoneal cavity with 2-3 ml of Gey's balanced salt solution containing 1:20000 heparin (La Motte Chemical Products Co., Baltimore). The mixture of ascitic fluid and physiologic solution was withdrawn aseptically and squeezed twice with a syringe through a 27-gauge needle to free the intracellular Toxoplasma. This suspension was centrifuged for 3 minutes at 500 r.p.m. to separate the remaining cells from the suspension of free Toxoplasma. The supernatant fluid was drawn off and the Toxoplasma were counted in a hemocytometer using physiologic saline solution as a diluent. Similarly, for the virulence studies 10-fold dilutions of the supernatant fluids of exudates containing a known number of Toxoplasma were injected intraperitoneally into groups of animals. The *macrophage cultures* were prepared and maintained as described earlier(3). Five to 7 days after the intraperitoneal injection of the chemotactic agent (glycogen or 12% sodium caseinate) the exudate was collected aseptically in Gey's balanced salt solution containing 1:20000 heparin. The number of cells in the suspension was determined with the hemocytometer. Aliquots of 9 ml of cell suspensions were prepared to contain 2000-2500 cells per mm^3 and poured into small Petri dishes, which had 10 small coverslips on the bottom. Usually the Toxoplasma were mixed with the cells at this time: to 9 ml of cell suspension one ml of Toxoplasma suspension, prepared in the manner described above, was added. The number of Toxoplasma in the suspension was adjusted to approximate in the final mixture a proportion of one Toxoplasma per 7 macrophages. In some experiments, the size of the inoculum was either increased or decreased. The Petri dishes were incubated at 37°C for one to 2 hours in an air-tight box filled with alveolar air. Each coverslip was then removed, washed in balanced salt solution, covered with a thin film of formvar, and finally placed in a small screw capped vial. Each vial contained 0.5 ml of culture medium with 30% serum (guinea pigs, rat, or rabbit) in Gey's solution. To inhibit the growth of contaminants, streptomycin was added in a final concentration of 2 μg/ml and penicillin 50 u/ml. These concentrations of antibiotics have been reported not to inhibit the multiplication of Toxoplasma(5). To adjust the pH to 7.2 the tubes received 5% CO_2 in air. The tubes were closed airtight and placed in the incubator in almost a horizontal position. Coverslips were taken out immediately after the period of sedimentation and phagocytosis, and at about 12-hour intervals. The cultures were fixed in absolute methyl alcohol for 10 minutes, then the formvar was removed mechanically from the back of the slide. After drying, the slides were placed in ethylene dichloride for one hour to dissolve or alter the remaining formvar. This procedure was found to be essential to obtain good staining of the macrophages and the Toxoplasma. The preparation was then washed in water and stained in hematoxylin for 2 hours, and mounted on slides with permount and covered with a coverslip.

Immunization. Rats and guinea pigs were immunized by subcutaneous injection of living Toxoplasma obtained from the peritoneal fluid of infected mice. One to 3 injections of 0.5 ml each were given at intervals of 3 weeks. For the first injection the peritoneal fluid was diluted 100-fold, for the second 10-fold and it was used undiluted for the third injection.



Various stages of intracellular multiplication of Toxoplasma within macrophage cultures from mice, guinea pigs and rats. Hematoxylin.

FIG. 1. Mouse cells, one hr. Indication of disappearance of one of the parasites from the cell. ×1800.

FIG. 2-4. Mouse cells, 67 hr. Macrophages with increasing numbers of parasites and rupture of the cell. ×1800.

FIG. 5. Mouse cells, 51 hr. Macrophage destruction around a ruptured cell. ×250.

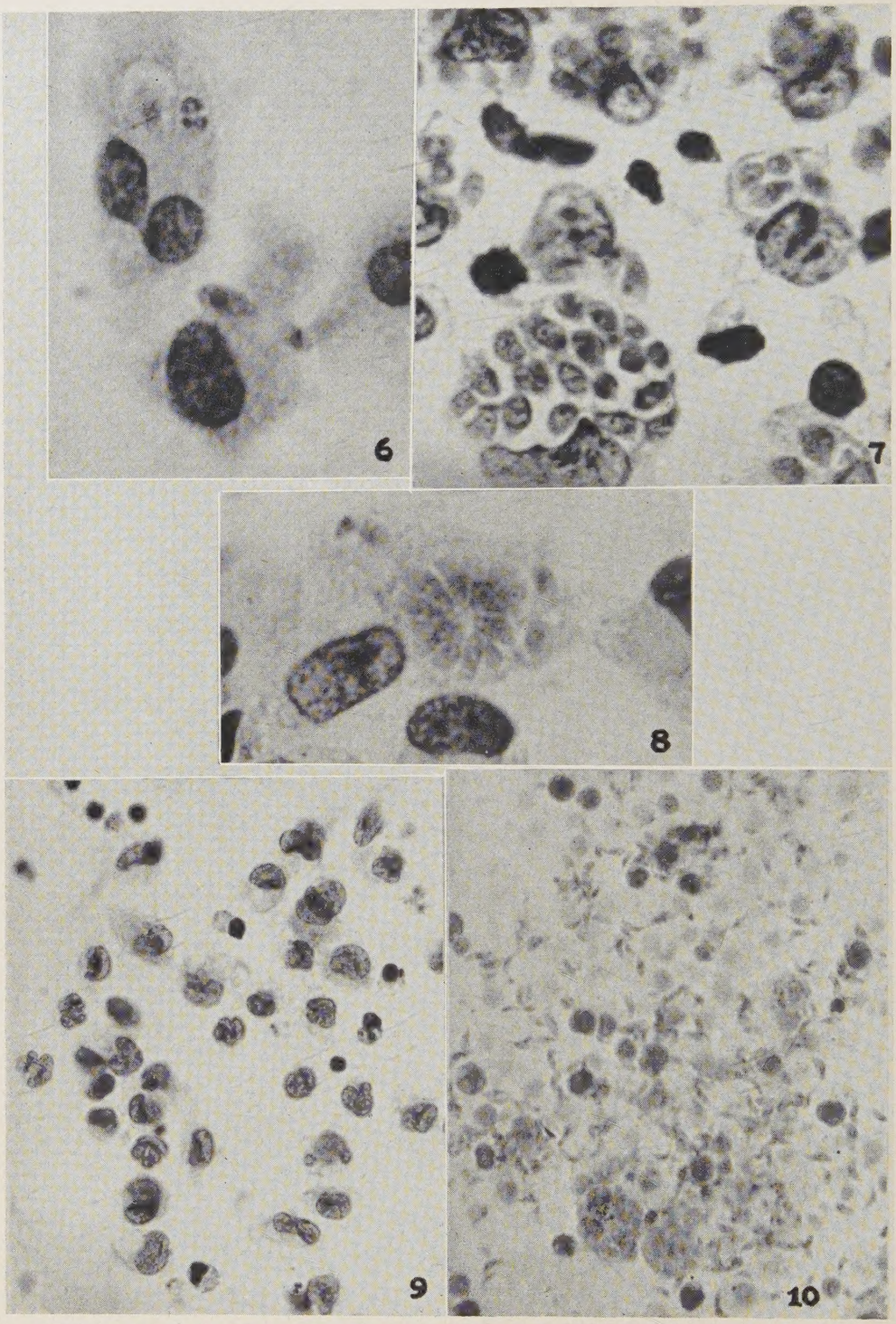


FIG. 6. Guinea pig cells, one hr. Upper cell with 3 parasites losing their staining properties. ×1800.

FIG. 7. Rat cells, 19 hr after heavy infection. Cells containing 4-20 or more parasites. ×1800.

FIG. 8. Rat cells, 51 hr. "Rosette" formation. ×1800.

FIG. 9 and 10. Macrophages from immunized and normal rat cultured for 72 hr in a medium containing immune and normal serum, respectively. Difference in the number of Toxoplasma in the cultures indicates the inhibitory property of the macrophages from the immunized rat. (Fig. 9.) ×600.

Simultaneously with the immunization, control animals were injected with 0.5 ml of a suspension of macrophages obtained from peritoneal washings of normal mice. As a criterion for the presence of antibody in sera obtained from immunized rats and guinea pigs, the dye-test for cytoplasm modifying antibody(6) was used.

Experimental observations. 1. *Multiplication of Toxoplasma in mouse macrophages.* At the beginning of cultivation the proportion of cells containing Toxoplasma and the number of parasites per infected cell depended on the size of the inoculum (Fig. 1). The first observed change within about 8 hours was an almost complete disappearance of stainable intracellular parasites. During this phase the cytoplasm of many parasites remained unstained and at the site of the nucleus a small structure, stained deeply with hematoxylin, was visible. A few hours later Toxoplasma reappeared within the cells and steadily increased in number. At 36 hours and later cells were found containing 1, 2, 3, 4, 6, 8 or more parasites, in general a multiple of 2 (Fig. 2 and 3). In some instances a vacuole was clearly visible around the Toxoplasma. Mouse monocytes contained rarely more than 16 parasites. In this stage, the nucleus of the cell was pushed towards the periphery and finally, the cell ruptured and released the Toxoplasma (Fig. 4). Because the culture was covered with formvar the parasites could not escape into the fluid but were held on the slides and were therefore phagocytized by other macrophages where they multiplied again. This process of cell rupture and reinfection finally resulted in the destruction of all the macrophages. In the early phases of cellular rupture, it could usually be observed that the release of Toxoplasma led to damage of adjacent cells although no parasites could be seen within these cells (Fig. 5). The release of free Toxoplasma depended on the size of the inoculum. Thus, in cultures in which the ratio of Toxoplasma to macrophages was 3:5 in the infection period, Toxoplasma was released from the cells after 19 hours of cultivation; with a ratio of 1:10 they only appeared after 43 hours. If the inoculum was very small (1:20), no increase of Toxoplasma was observed over a period of 6 days.

TABLE I. Per Cent of Intracellular Toxoplasma in Macrophages from Mice, Guinea Pigs, Rats and Rabbits after Increasing Length of Incubation.

	Min. of incubation		
	15	30	45
Mouse	38	70	67
Guinea pig	82	93	96
Rat	63	70	77
Rabbit	77	100	100

2. *Multiplication of Toxoplasma in macrophages of different species.* When cultures of macrophages from mice, guinea pigs, rats, and rabbits were set up side by side, a number of differences were observed in the rate of uptake of Toxoplasma by the cells and of multiplication of the parasites and in the extent of destruction of macrophages resulting from the increasing number of intracellular parasites. Because humoral factors possibly present in peritoneal exudates and influencing the interaction between macrophages and Toxoplasma had to be excluded, the procedure of preparing the cultures had to be modified. The macrophages were first allowed to settle on the slides during an hour's incubation, which were then washed in physiological solution, placed in new Petri dishes and covered with a suspension of Toxoplasma. After a second period of incubation for one hour the cultures were finally washed and covered with formvar and cultivated as described. From Table I it is clear that first the parasites were taken up more rapidly and more completely by macrophages from rats, guinea pigs, and rabbits, than from mice. Secondly, the disappearance of intracellular Toxoplasma in the first few hours of cultivation was observed consistently and to a more complete extent in the cultures of macrophages from guinea pigs,

rats, and rabbits than in those from mice (Fig. 6). Thirdly, in cultures from the guinea pigs, rats, and rabbits a longer time elapsed until the parasites began to reappear compared with those of mouse macrophages. Likewise intracellular multiplication was much slower in macrophages from rats, guinea pigs, and rabbits. In consequence, the cultures were destroyed to a far less extent and only after prolonged cultivation. It was also observed that mouse cells seemed far more susceptible to intracellularly accumulating parasites than the cells from other animals. This resulted in a more rapid destruction of the cultures of mouse cells, whereas a high number of Toxoplasma accumulated within cells of rats and guinea pigs (Fig. 7 and 8). Comparing the susceptibility of these species to the intraperitoneal infection with Toxoplasma, it was found that mice and guinea pigs were killed by approximately 5 to 10 parasites whereas rats survived the injection of 6×10^6 Toxoplasma. Mice were also susceptible to small numbers of parasites injected subcutaneously whereas guinea pigs were much more resistant to this route of infection.

3. *Multiplication of Toxoplasma within macrophages from immunized animals, and the role of antiserum.* Because of the high virulence of the RH-strain for mice, immunization could not be achieved and the experiments were done with macrophages from rats and guinea pigs previously immunized with living Toxoplasma. Sera from normal animals never showed a titer of cytoplasm modifying antibodies above 1:8, whereas the sera from immunized animals had titers from 1:2000 to 1:8000. These titers correspond well with those given in the literature(6). To separate inhibitory properties residing within the macrophages from those exhibited by the serum, macrophages from normal and immunized animals were cultivated separately in normal and in immune serum. In addition, in some experiments, the macrophages were washed with balanced salt solution before they were infected with Toxoplasma to remove as completely as possible any antibody absorbed on the surface of the cells. Extent of multiplication of Toxoplasma in such cultures, as judged by the accumulation of intracellular and free parasites, is summarized in Table II. Macrophages from immune animals cultivated with immune serum exhibited the greatest inhibitory property, and after 4 days of cultivation only a few intracellular Toxoplasma could be found (Fig. 9 and 10) at a time when in the cultures of macrophages from normal animals free and intracellular Toxoplasma were abundant. Less inhibition was exerted by either immune cells with normal serum or vice versa. Washing the macrophages before exposing them to the Toxoplasma did not abolish their limited inhibitory power. Pycnotic nuclei, presumably from dying Toxoplasma, were frequently observed within macrophages from immunized animals cultivated with immune serum.

TABLE II. Degree of Intracellular Multiplication of Toxoplasma within Macrophages Derived from Immunized Animals and Influence of Immune Serum.*

Cells: / Serum:	Immune / "	Immune / Normal	Normal / Immune	Normal / "
Exp.† 37	1	2	2	4
43	1	2	2	4
61	1	3	2	4
76	1	2	2	4
81	1	2	3	4
84	1	2	2	4
91	1	2	3	4
94	1	2	2	4

* Multiplication is judged by the presence of free and intracellular parasites and is rated on an arbitrary scale, 4 indicating maximal and 1 minimal multiplication.

† Exp. 37-84 were done using rat macrophages, 91 and 94 guinea pig cells.

Discussion. The results reported indicate that intracellular reproduction of Toxoplasma in macrophage cultures occurs in 3 phases: penetration and phagocytosis, multiplication, and release of parasites followed by infection of new cells. There was good evidence that macrophages do phagocytize Toxoplasma which themselves penetrate actively into nonphagocytic parenchymal cells. The phagocytic activity of the macrophages was found different with cells from mice, rats, and guinea pigs. It was consistently observed that a few hours after infection only very few cells contained stainable Toxoplasma although a great number of parasites were present immediately after infection. The significance of this disappearance is not clear. Sabin and Feldman(6)

described similar changes of the staining properties under the influence of heat, cold, and antibody, suggesting that such changes are manifestation of degeneration. Differences of behavior of Toxoplasma within macrophages from various species, as time of reappearance of stainable forms and susceptibility of the cells to the accumulating parasites, suggest that the interaction between macrophages and Toxoplasma is a determining factor in the mechanism of native resistance. This view has been raised by Frenkel(7) and is supported by evidence presented in this paper. Finally, our experiments indicate that immunization results in restriction of intracellular multiplication of Toxoplasma by the macrophages as well as by a humoral factor. It should be added that it is difficult to prove conclusively that the inhibition exerted by cells was not due to remaining antibody adhering to the cells, although washing of the macrophages did not abolish this inhibition. However, the fact that macrophages from immune animals combined with immune serum were much more inhibitory than the immune serum alone with normal macrophages, supports the assumption of a cellular factor in immunity against Toxoplasma. This duality had been postulated much earlier by Levaditi (8) and again by Frenkel(7). This property of macrophages from immune animals to inhibit intracellular proliferation of Toxoplasma is no unique observation. Thus, destruction of malarial parasites by spleen macrophages in immunized animals has been described long ago(9), and similarly, inhibition of intracellular multiplication of tubercle bacilli by macrophages from animals vaccinated with BCG has been demonstrated in tissue cultures(10), but the mechanism of cellular inhibition in any of these infections remains unknown.

Summary. *Toxoplasma gondii* has been propagated successfully in macrophage cultures of adult laboratory animals. The pattern of the interaction between the macrophages cultured *in vitro* from different animal species could be correlated with the susceptibility of the species to the infection with Toxoplasma. Macrophages cultured *in vitro* from animals actively immunized by injection with living Toxoplasma had an inhibitory effect on the intracellular multiplication of these parasites. This effect was increased by the presence of immune serum which had a high titer of cytoplasm-modifying antibodies.

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Radiation Effects on Pneumococcal Infection Produced by Subcutaneous Injections into White Mice.* (21118)

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X-irradiation in doses of 400 to 600 r has been shown to produce a fatal bacteremia in white mice(1,2). This is apparently due to an impairment of the blood clearing mechanisms which normally destroy bacteria that occasionally pass through the intestinal wall. A recent review of the physiological changes brought about by irradiation has been made by Taliaferro(3). It has also been shown that irradiation decreases the resistance of animals to experimental(4-9) and natural infections(10,11), and that resistance is related to the post-irradiation time of the infection. The relation of this to changes observed in the blood and certain organs of irradiated animals is not entirely clear(5,8).

One approach to the evaluation of the effect of irradiation on the local defenses, as well as on the systemic defenses, is the determination of the time required for bacteria to reach the blood stream from a subcutaneous injection. Furthermore, this procedure simulates conditions accompanying an atomic blast, in which infectious agents could be carried through the skin with flying debris, and may indicate whether an increase or decrease in the severity of such infections could be expected. For this purpose the experiments reported in this paper were designed.

Methods. Female white mice, 6 to 11 weeks old, average weight 20 g, obtained from the Rockland Farms, were used. They were observed for at least 5 days after receiving, in order to have all test mice in a good nutritional state and free from disease. Small groups of 10 to 20 mice were injected subcutaneously with 0.2 ml of a suspension of Pneumococcus Type III made in pH 7 buffer from a 15- to 18-hour growth on a blood agar slant. The turbidity read 55 on a Klett Summerson colorimeter and the suspension contained approximately 2,000,000 bacteria per ml. The dosage used had been found to kill most of the mice in a 72-hour period. One-half of each group were subjected to total body x-irradiation in a single exposure delivered for 19 minutes at 220 Kvp, 15 ma., at a distance of 85 cm using ½ mm copper and one mm aluminum filters. This dosage was 350 r delivered on the skin as measured by a Victoreen r meter. Twelve mice at a time were rotated slowly through the field during exposure. The apparatus used for this purpose was essentially the same as that described by Miller *et al.*(1). This dose was sublethal for mice as determined by many other investigators and reaffirmed in our laboratory. Twice this dosage was 100% lethal. The injection of organisms was made with different groups of mice at zero time, 3 days and 6 days after irradiation. In order to study the time from injection to the appearance of bacteria in the blood, and also the time from bacteremia to death, tails were clipped and 3 drops of blood from each inoculated onto blood agar plates at intervals of 15 minutes for the first hour, at 2-hour intervals for the first 8 hours, and at 6- to 12-hour intervals thereafter until death. Plates were incubated for 48 hours and examined both grossly and by staining smears from each colony. Each mouse was marked so it could be studied individually. The pneumococcus was used because it produced a readily identifiable bacteremia in mice.

Results. Injection given immediately after irradiation. Each of 46 mice were studied individually in groups of 10 to 12 as outlined above. One-half of the group were irradiated and the other half served as controls. All were injected with pneumococci immediately

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TABLE I. Effect of Irradiation Given 3 and 6 Days before Infection of Mice with Pneumococci.

	% with bacteremia					% dead after bacteremia			
	I		II			III		IV	
	3 days		6 days			3 days		6 days	
Hr	Non-irrad. (23)	Irrad. (23)	Non-irrad. (28)	Irrad. (27)	Hr	Non-irrad. (23)	Irrad. (23)	Non-irrad. (28)	Irrad. (27)
1	13	43	19	15	10*	9	13	3.5	18.5
4	35	52	19	15	15	17	17	7	44.4
8	43	65	22	15	20	30	35	11	52
24	65	95	42	37	25	65	70	38	63
48	87	100	56	73	30	87	87	61	85

No. in parentheses are total animals used.
* Refers to the hours from the time bacteremia was established until death.

after irradiation. There were rather wide variations within both groups in the time of death, and the time that bacteria appeared in the blood. However, the time from the appearance of an extensive bacteremia until death occurred was quite constant (about 25 hours) in both the control and irradiated groups. The time from injection to the appearance of either a few or many bacteria in the blood was about the same in the irradiated as in the control mice.

Injection given 3 days after irradiation. A total of 60 mice in 5 groups of 12 each were studied as before, but the injection was given 3 days after irradiation of one-half of the group. Again it was found that time from inoculation to death and to the finding of bacteria in the blood varied widely in individual mice, while the time from extensive bacteremia to death was more constant. A greater percentage of the irradiated mice were dead at any given time than were the controls. Table I, which includes only a portion of the observations made, indicates that irradiation decreases the time for inoculation of the organisms under the skin until they appear in the blood in large numbers. More of the irradiated mice showed extensive bacteremia at any given time than did the non-irradiated. The statistical assurance for a real, rather than apparent, reduction in the time to extensive bacteremia was afforded by using the one-sided Wilcoxon test(12). (This test was utilized since its validity is not dependent upon the form of the distribution.) A value, $P = 0.012$, was found for the data in Column I. Column III indicates that the time from extensive bacteremia to death was little influenced by irradiation given 3 days before infection.

Injection given 6 days after irradiation. Fifty-five mice were studied under these conditions. They were divided into 4 groups. One-half of each group had been irradiated 6 days before infection. Column II, of Table I, shows that irradiation given 6 days before inoculation had little effect on the time for bacteremia to occur. The most striking difference is shown by the values in Column IV. The time from bacteremia to death was significantly decreased in the irradiated mice ($P < 0.005$ by the test noted above). Fifty-two per cent of the irradiated mice died in 20 hours or less after an extensive bacteremia was observed. Only 11% of the controls died within this time period. At almost any given time after bacteremia was established there was a considerably greater percentage of the irradiated mice dead.

Discussion. Irradiation at the time of infection had little effect on the susceptibility of mice to the pneumococcal infection. However, it increased their susceptibility when the infection was produced 3 and 6 days after irradiation. Similar observations have been made with other bacteria introduced into mice by other routes(5,8). At 3 days, pneumococci reached the blood stream in a shorter time in the irradiated animals, but the time from bacteremia to death remained the same. This could mean that at this period the local defenses are lowered, but the blood clearing mechanisms have not been effected. Six days after irradiation there was no difference in the time required for the establishment of bacteremia, but the time from bacteremia to

death was decreased in the irradiated animals. This indicates possible repair of the local defenses, but an impairment of the bactericidal and/or filtering mechanisms of the blood. It is recognized that the irradiated animals may have had less ability to withstand the bacteremia at this time because of other changes induced by the irradiation.

Irradiation 48 hours prior to inoculation has been reported to damage lymphatics so that bacteria reached the blood stream more rapidly(8). The induced bacteremia was no more lethal in the irradiated animals. This agrees with our results, at 3 days, with a more fulminating infection. The increased lethality of the bacteremia at 6 days may possibly be explained by recent reports of a lowered bactericidal activity of the blood in rabbits(13) and rats(14), 6 days after irradiation.

The present data imply altered responses of resistance to infection as a function of post-irradiation time. However, the type of animal, bacteria, and irradiation involved, as well as the dose used, may materially alter the character of the phenomena—at least the temporal relationship.

Summary. Whole body x-irradiation of white mice with 350 r influenced an infection with pneumococci induced by subcutaneous inoculation as follows: 1. Little or no effect was observed when radiation exposure was given at the same time as the inoculation of the bacteria. 2. The time from inoculation to death, and the time required for bacteremia to become established, was reduced when radiation exposure was given 3 days prior to inoculation. The time from bacteremia to death was not changed. 3. The time from inoculation to death, and the time from bacteremia to death, was reduced when radiation exposure was given 6 days prior to inoculation. The time required for bacteremia to become established was not changed.

The authors would like to express their thanks to Dr. Jack W. Grossman of the Department of Radiology of the Lovelace Clinic for irradiating the mice, and to Mr. Julian P. Henry of the Lovelace Foundation for building the turntable on which the mice were rotated while being irradiated. We are also grateful to Dr. J. L. Hodges, Department of Mathematics and Statistics, University of California, Berkeley, for his advice as to an appropriate statistical test for the data.

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Neural Mechanisms in Sexual Behavior. I. Reflexology of Sacral Segments of Cat.*† (21119)

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Most analyses which have been made on conduction and integration in the spinal cord have been based on those spinal mechanisms which are related to locomotion. Less information is available concerning the properties of the centers serving non-locomotor behavior. Sexual reflexes are an example of such and they exhibit features which deviate considerably from the better known cord mechanisms. There is an absence of the proprioceptive component of the segmental reflex. There is a conspicuous crossed ventral root outflow, a feature missing in the lumbo-sacral intumescence. It is the purpose of this report to describe some of the phenomena related to the genital innervation of the cat and to analyze the properties of the caudalmost segments of the spinal cord.

Material and methods. A series of 18 cats was prepared with dial or nembutal anesthesia and used for stimulating and recording experiments of the peripheral nerves, nerve roots, or spinal cord. In some of the animals, transection in the lower thoracic levels preceded the experiments. This had no discernible effect on the reflex phenomena. Silver wire electrodes were used for stimulating and recording from the nerves and nerve roots. The potentials of the spinal cord were obtained with glass coated silver solder micro-electrodes. Both males and females were included in the series.

Observations. Stimulation of the S_2 dorsal root evokes a response on the corresponding ventral root (Fig. 1*a*) which resembles, in its components, the segmental reflexes of the first sacral segment and of the caudal few lumbar segments. This similarity is undoubtedly due to the fact that the second lumbar segment has extensive innervation of locomotor structures. There is a proprioceptive spike, the 2-neuron reflex, followed by a dispersed cutaneous response (the flexor reflex of various authors). A complete absence of a crossed response further substantiates the relation of this segment to the segments cranial to it and raises the question of its innervation in any midline muscles.

The third sacral segment shows an abrupt change in properties from the second and more cranial segments. Stimulation of the dorsal root evokes a response on the corresponding ventral roots (Fig. 1*b*) which lacks the proprioceptive component completely. The cutaneous response is present and resembles that of the S_2 segment in form though remarkable in its great latency which is usually around 4.0 msec. The problem of comparison of cutaneous responses preceded by proprioceptive spikes with those in which the early component is lacking is one which is full of pitfalls(2) and this additional central delay of the cutaneous response is not necessarily indicative of a more complex pathway than in the other segments.

A feature lacking in the S_2 segment and conspicuous in S_3 is the crossed reflex. Stimulation of a dorsal root of the S_3 segment evokes a response not only in the corresponding ventral root but also in the opposite ventral root (Fig. 1*c*). The form of the response is similar to that on the homolateral side, but the amplitude is usually decreased. The latency is greater, varying from an additional several tenths of a millisecond to as much as 3 msec.

The first caudal (Ca 1) segment shows a pattern of segmental response different from its predecessor. It shows a proprioceptive spike which gives it the appearance of the segmental response of the lumbar region (Fig. 1*d*). Following the spike is a distinct cutaneous component. The crossed reflex (Fig. 1*e*) lacks the proprioceptive spike and has a

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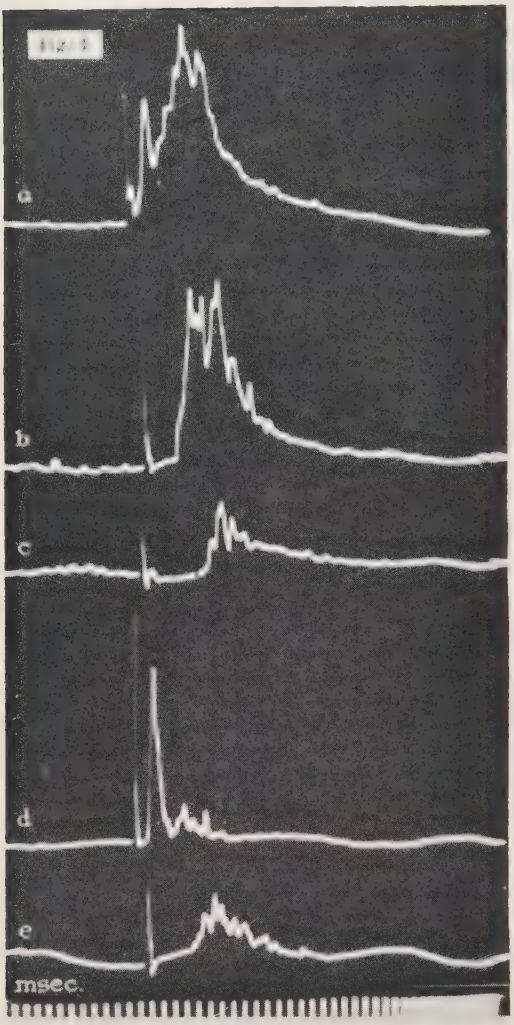


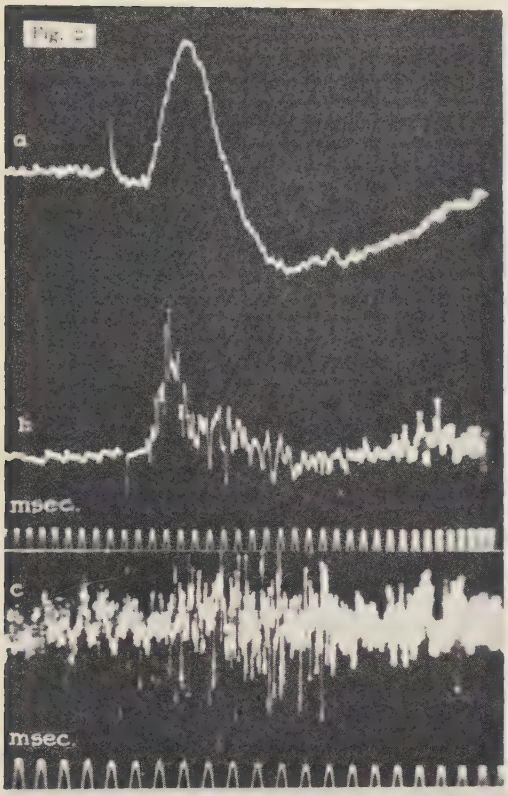
FIG. 1. *a*. Response on S_2 ventral root from stimulation of ipsilateral S_2 dorsal root. *b*. Response on S_3 ventral root from stimulation of ipsilateral dorsal root. *c*. Same from stimulation of contralateral S_3 dorsal root. *d*. Response on Ca_1 ventral root from stimulation of ipsilateral dorsal root. *e*. Same from stimulation of contralateral dorsal root.

later and more continuous cutaneous response. However, the remarks above, relevant to the interpretation of the cutaneous component when complicated by the preceding firing of the neurons in a proprioceptive spike, are also relevant here.

The total latency of the proprioceptive spike is less than 1.5 msec. and exhibits a central latency consonant with the 2-neuron reflex. The cross response commences at 4.0 msec.

The signal delivered to the spinal cord from stimulation of the genitalia operates within the segmental system described above. Apparently in this species the principal innervation of the penis and vulvar vestibule is from the S_3 segment. Stimulation of the penis gives a marked dorsal cord potential (Fig. 2*a*) which is confined to that segment. In this record, the conduction spike does not show, probably because of the dispersed nature of the volley. The latency of the dorsal cord negativity is 3.2 msec. Placing the electrodes on the intact S_3 dorsal root in the same experiment (Fig. 2*b*) shows a volley (resulting from a single condenser discharge across the intact penis) which shows considerable dispersion commencing at 2.0 msec. In another experiment, a record made from the peripheral end of a cut pudenal nerve yielded a potential which begins at 1.2 msec., and continues for at least 6 more msec. The complex form indicates several groups of fibers were participating. The dispersion is more clearly illustrated in Fig. 2*c*. This was a female cat. The stimulating electrodes were placed in the vulvar vestibule and, from a small filament of the S_3 dorsal root, the activity following a single shock to the vestibule was recorded. Because of the recording conditions, much of the activity is shown as single unit spikes. The considerable dispersion of the volley is illustrated. In response to stimulation of the genitalia, a reflex on the S_3 ventral root is evoked. In Fig. 3*a* and *b*, the dorsal roots on the left side were cut. The response on the right, thus, was an ipsilateral reflex. The total latency is about 8.0 msec. of which 3.2 msec. may be taken as the afferent conduction time. The outflow potential rises to a rapid peak and terminates within 4.0 msec. after its commencement. As it ceases before the afferent volley has ceased, some quenching mechanism is indicated. There is no sign of a proprioceptive component. The crossed reflex in the S_3 ventral root following stimulation of the penis or vestibule resembles the uncrossed outflow described above. The latency is from 0.6 msec. to 2.0 msec. more than that of the uncrossed reflex.

From similar stimulation of the genitalia, no response is to be obtained from the S_2



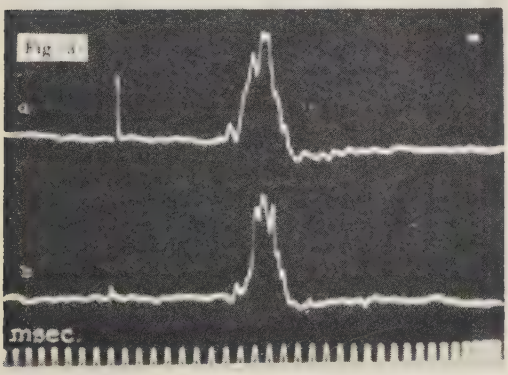


FIG. 2. *a*. Male cat. Response at dorsum, of cord, S_3, following stimulation of penis. *b*. Record from S_3 dorsal root, stimulus as in *a*. *c*. Response on S_3 dorsal root, following stimulation of vulva.

FIG. 3. *a*. Response on S_3 ventral root on intact side from stimulation of penis. *b*. Response on opposite side.

segment but the Ca_1 segment shows a small return, both crossed and uncrossed. Because of the nature of the records the onset is not easily fixed, but it seems to be slightly earlier than in the preceding segment.

The exact modalities involved in these responses are not apparent from the dorsal root stimulation. To make some test of this, records from the S_3 dorsal root were made on a cat with stimulation of the penis in its retracted position (with the stimulating electrodes in the preputial cavity), on the glans of the everted penis, and on the anus. This shows a latency of 2.5 msec. from the glans penis as contrasted with a latency of 1.8 msec. in the response to stimulation of the prepuce as well as the glans. With the stimulating electrodes within the anus, the corresponding delay drops to 0.9 msec. Between the first 2 figures, some of the discrepancy may be due to the increase conduction distance, certainly less than 2 cm. Probably the greatest factor in these different latencies, is the different spectrum of fiber size concerned in each instance. A question is raised by these figures concerning the muscle sense of the rectal and preputial sphincters. While there is no characteristic 2-neuron spike in this segment, as mentioned above, the possibility that the faster component of the pudenal nerve may be related to some kind of muscle sense must be entertained. The relatively smaller amplitude of the response from the glans probably indicates a rather restricted population of fibers as one might expect from the specialized nature of the organ.

Discussion. The activity described in these experiments is a part of the behavior pattern of the genitalia. It is not specifically concerned with sexual behavior, *per se*, as the genitalia have other behavioral aspects. The properties of the reflex phenomena described resemble the so-called "flexor" reflex—the cutaneous component of the segmental return. This is usually thought to be protective in its function, and may in fact be considered in this instance. It resembles the "flexor" reflex also in form and in its lengthy central time. It is not consistent with our knowledge of the behavioral meaning of the reflex phenomena to identify a given simple response with a particular behavioral entity. As locomotion is not a simple sequence of proprioceptive or cutaneous or crossed extension reflexes, but an integrated behavior utilizing the mechanisms serving these physiological fragments, in the

same sense, the behavior of the genitalia is not to be elucidated by the study of a simple reflex. The phenomena serve to define the properties of the pathways which mediate the activity of the genitalia.

The lack of the proprioceptive reflex from the segmental reflex of S_3 indicates that the striated musculature of the genitalia does not have a myotatic regulation with the same properties as that of the limb or tail muscles. Whether there are different types of muscle end organs, or any muscle end organs at all, must remain questionable until a histological examination has been made.

Summary. Activation of the third sacral and first caudal segment of the spinal cord evokes a segmental response on the ventral root resembling the cutaneous component of the segmental reflex of the more cranial parts of the spinal cord. A proprioceptive spike is absent in S_3, present in Ca_1. In each of these segments, there is a crossed reflex return resembling the cutaneous component and frequently somewhat later and smaller than the ipsilateral response. Stimulation of the penis or of the vulvar vestibule elicits the same response, though without the proprioceptive spike in the Ca_1 ventral root. Spinal transection in the lower thoracic region does not alter these reflexes under the experimental conditions.

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Thyroid Feeding and Vitamin B_6 Deprivation in the Rat.* (21120)

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In an earlier investigation(1) it was found that the basal metabolic rate of vit. B_6-deprived rats was not different from that of pair-fed control animals although lower than that of *ad libitum*-fed controls when expressed as oxygen consumption per unit of body weight. There is considerable evidence that the efficiency of energy production is altered by vit. B_6 deprivation. However, attempts to lower the basal metabolic rate by administration of thiouracil or by thyroidectomy did not cause definite alterations in the syndrome of vit. B_6 deprivation(1). It was felt advisable to study the effects of a hyperthyroid state on the deprivation syndrome in rats. The results of this study are presented.

Methods. Forty albino rats of the Wistar strain and of both sexes were divided into 4 groups equal with respect to number, sex distribution and an initial average body weight of 103 g. All animals were housed in individual, screen-bottomed cages with drinking water freely available. The basal diet employed was the 20% casein, 20% corn oil, vit. B_6-free diet previously described(2). Two groups were further provided with 50 μg pyridoxine hydrochloride per rat per day in their food and served as control animals. One vit. B_6-deprived and one control group were given one mg desiccated thyroid (U.S.P., Nutritional Biochemicals) per g of diet. To eliminate differences in analytical results between groups consequent to differences in the amount of food consumed, all groups were pair-fed with the vit. B_6-deprived group given desiccated thyroid such that the average daily food consumption of all groups was 13.4 g per rat. Following an experimental feeding period of 21 days, the animals were fasted for 18 hours and anesthetized with an intraperitoneal injection of 2% butylone in 0.9% saline. Blood was removed from the exposed hearts with the aid of a hypodermic syringe and was heparinized and pooled for each group. The following analyses were done in duplicate on

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TABLE I. Effects of Feeding Desiccated Thyroid to Vit. B_6-Deprived and Pair-Fed Control Rats (10 Rats/Group).

Group	Avg body wt gain, g	Carcass total crude fatty acids, %	Blood packed cell vol, %	Blood hemoglobin, g %	Blood urea, mg %	Blood sugar, mg %	Blood amino N., mg %
Control	89	13.3	42	12.1	24.0	106	11.9
Deprived	73	9.9	44	12.5	34.0	107	14.2
Control + thyroid*	70	7.8	44	11.3	23.8	80	14.6
Deprived + thyroid*	55	5.5	45	11.9	22.4	70	14.3

* Signifies provision of U.S.P. desiccated thyroid 0.1% in the diet.

the pooled blood samples by the procedures stated: hemoglobin, Collier(3); urea, Archibold(4); sugar, Nelson(5); amino nitrogen, Frame *et al.*(6); and packed cell volumes by the standard procedure. Carcasses were pooled for each group, frozen, passed through a power grinder, homogenized and analyzed for total crude fatty acids(7).

Results. The results of this study are set down in Table I. Following 21 days of experimental feeding, none of the animals exhibited acrodynia, the external manifestation of vit. B_6 deficiency in the rat although the deprived animals, particularly those fed desiccated thyroid, were in a poor, unkempt condition. As shown in the data, those animals not provided with pyridoxine had subnormal body weight gains on comparison with their comparable controls. As would be expected, desiccated thyroid-feeding *per se* reduced the body weight gains of both deprived and control rats. Carcass total crude fatty acid levels show the same pattern as body weight gains. Vit. B_6 restriction and thyroid-feeding each diminished carcass total crude fatty acids, the effect being greatest when both occurred concurrently. The customary difference(8) in total crude fatty acid levels of deprived and control animals was evident even when thyroid was administered.

The results of blood analyses as shown in Table I indicate that neither vit. B_6 deprivation nor thyroid-feeding markedly altered hemoconcentration. Slightly lower hemoglobin levels were noted in those animals fed thyroid but it cannot be stated that this necessarily represents anemia. A consistent observation in this laboratory has been an elevated fasting blood urea level in vit. B_6-deprived rats(2) which has been attributed, on the basis of *in vitro* studies, to an increased rate of production by liver tissue(9). In the present experiment this elevation is reconfirmed. However, with dietary provision of desiccated thyroid, the fasting blood urea level of deprived animals was restored to normal although that of controls was not altered. It is of interest that thiouracil administration and thyroidectomy did not eradicate this difference but rather elevated the fasting blood urea levels in both deprived and control groups(1). Further, thyroid-feeding depressed fasting blood sugar levels in both groups which might be indicative of the general catabolic state suggested by the data on body weight and carcass fat. No marked effect of vit. B_6 deprivation on fasting blood amino nitrogen levels was noted which is in accord with earlier observations(2). Feeding of desiccated thyroid was without apparent effect on blood amino nitrogen levels.

The dosage of desiccated thyroid used in this study was sufficient to cause decreased body weight gains and to markedly lower the amount of carcass fat in the experimental animals. Moreover, the dosage was similar to that which Houssay(10) demonstrated to be sufficient to counteract the effects of thyroidectomy and which Weiss *et al.*(11) showed to be non-toxic for rats. Cursory examination of the animals in handling showed them to be hyperexcitable which again suggests that a hyperthyroid state was attained under these experimental conditions.

The earlier investigation(1) of thiouracil administration and thyroidectomy demonstrated that the biochemical defects of vit. B_6 deprivation are not altered by hypothyroidism. The present study suggests the same conclusions for hyperthyroidism. Thus these

biochemical defects are apparently not caused or prevented by alterations in thyroid activity. This suggestion is supported by the finding of a normal basal metabolic rate in deprived animals on comparison with pair-fed controls(1).

Summary. On administration of desiccated thyroid in the diet, the syndrome of vit. B_6 deprivation in the rat was not markedly altered. One apparent effect of thyroid-feeding was to eliminate the customary elevation in fasting blood urea consequent to vit. B_6 restriction. This and an earlier study suggest that the biochemical defects of vit. B_6 deprivation in the rat are not caused or prevented by alterations in thyroid activity.

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Prolongation of Serum Prothrombin Time After Heparin. (21121)

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In the performance of the prothrombin consumption test the prothrombin time is determined 15, 30, 45, and 60 minutes following coagulation and centrifugation(1). The prothrombin time of *freshly* coagulated and centrifuged blood is not considered a true measure of prothrombin since considerable free thrombin occurs in the serum(1). The antithrombic effect of heparin was, therefore, expected to influence the prothrombin time of fresh serum. In order to confirm this hypothesis, a comparison of the prothrombin consumption tests was carried out before and after administration of heparin, with particular emphasis upon the prothrombin time of fresh serum. Further evidence of a thrombin effect as well as thromboplastin effect in fresh serum was obtained by the interaction of calcified serum upon plasma at various time intervals before and after administration of heparin.

Material and methods. Twenty normal individuals were divided into 2 groups of 10 each. Prothrombin consumption tests according to the procedure of Quick(1) were performed in all 20 individuals just before and one hour after the intravenous administration of heparin. The first group of 10 individuals received 50 mg of heparin while the second group received 75 mg of heparin each. Deprothrombinized oxalated plasma was obtained with barium sulfate according to Rosenfield and Tuft(2). In view of the emphasis in this study upon the prothrombin time of fresh serum the tests were not performed at intervals of 15, 30, 45, and 60 minutes following coagulation as described by Quick, but immediately after coagulation (zero-hour) as well as 30, 45, and 60 minutes later. A. *Prothrombin consumption test.* Five ml of venous blood were placed into a test tube in a water bath at 37°C. When coagulation was complete the tube was centrifuged for one minute at top speed. The prothrombin time of the

serum was determined immediately thereafter by adding 0.1 cc of the supernatant serum to a mixture of 0.1 cc of deprothrombinized human plasma, 0.1 cc of Difco thromboplastin and 0.1 cc of 0.02 M calcium chloride. Following this "zero-hour" determination, the procedure was repeated at the above intervals before and one hour after the administration of heparin. B. *Interaction time of calcium, serum and plasma.* Equal amounts of 0.1 cc each of serum, obtained at zero-hour, 30, 45, and 60 minutes after coagulation, and 0.02 molar solution of calcium chloride were incubated in a small cuvette (75 x 6 mm) at 37°C for 3 minutes. Similarly, homologous plasma was incubated. Subsequently, 0.1 cc of the plasma was swiftly blown into the cuvette which had been placed into the microadapter of a Coleman Spectrophotometer Junior Model 6A. At this time a stopwatch was clicked. When the galvanometer light-beam began to swing to the left indicating the onset of coagulation, the watch was stopped. The physical setup of this procedure was identical to that utilized in the photoelectric determination of the plasma prothrombin time (3). The only difference consisted in the fact that fresh serum was utilized instead of thromboplastin and that calcium was not added as a last step, but at an earlier phase of the procedure. The interaction time between calcium, serum and plasma was determined at the same intervals as described above, namely at zero-hour, 30, 45, and 60 minutes after coagulation. The procedure was carried out in a total of 20 individuals before and one hour after the intravenous administration of heparin, at which times serum and homologous plasma were derived from the same specimen of blood. Ten subjects received 50 mg of heparin while the other 10 received 75 mg of heparin. C. *Interaction of fresh serum and fibrinogen solution.* Normal serum obtained at zero-hour was added in the quantity of 0.1 cc to an equal amount of bovine fibrinogen (Chilcott-Warner), containing 300 mg % in physiological saline. Six determinations were carried out at 37°C. D. *Interaction of serum and homologous plasma.* Normal serum was added in the quantity of 0.1 cc to an equal amount of homologous citrated plasma. Six experiments were carried out at 37°C and repeated at 30, 45, and 60 minutes after coagulation.

TABLE I. Prothrombin Consumption Tests.

a) Before heparin admin.

Min. after coagulation	Mean value, 20 individuals (sec.)	Range (sec.)
*	2.3	1.8- 3.0
30	9.1	6.4-12.3
45	11.3	8.0-15.7
60	14.7	9.6-21.3

b) One hr after intrav. admin. of 50 mg heparin

Min. after coagulation	Mean value, 10 individuals (sec.)	Range (sec.)
*	8.1	5.2-13.0
30	14.5	11.8-16.0
45	18.1	16.0-21.3
60	20.9	17.6-26.5

c) One hr after intrav. admin. of 75 mg heparin

Min. after coagulation	Mean value, 10 individuals (sec.)	Range (sec.)
*	11.9	8.0-16.8
30	17.6	13.8-27.4
45	23.0	18.8-29.5
60	28.9	20.8-32.0

* Immediately (zero-hr).

Results. A. *Prothrombin consumption test.* As seen from Table I the prothrombin time obtained with serum of freshly coagulated blood increased from a mean value of 2.3 seconds at zero-hour to 9.1, 11.3, and 14.7 seconds at 30, 45, and 60 minutes after coagulation, respectively. While the prothrombin time at zero-hour had a range of 1.8-3.0 seconds only, the values were more widely scattered at 30, 45, and 60 minutes after coagulation. Following the intravenous administration of 50 mg of heparin the mean prothrombin time of fresh serum (zero-hour) increased to 8.1 seconds with a range from 5.2-13.0 seconds. Subsequent determinations 30, 45, and 60 minutes later, produced mean values of 14.5, 18.1, and 20.9 seconds, respectively. Upon intravenous injection of 75 mg of heparin, the mean serum prothrombin times increased further to 11.9, 17.6, 23.0, and 28.9 seconds at zero-hour, 30, 45, and 60 minutes after coagulation, respectively. B. *Interaction*

TABLE II. Interaction Time of Calcium, Serum and Plasma.

a) Before heparin admin.

Min. after coagulation	Mean value, 20 individuals (sec.)	Range (sec.)
*	9.4	6.8-12.0
30	12.6	10.0-17.0
45	14.3	11.2-20.0
60	15.8	12.0-20.9

b) One hr after intrav. inj. of 50 mg heparin

Min. after coagulation	Mean value, 10 individuals (sec.)	Range (sec.)
*	23.7	13.3- 35.7
30	36.4	15.8- 60.6
45	66.6	19.0-150.0
60	—	19.0- ∞

c) One hr after intrav. inj. of 75 mg heparin

Min. after coagulation	Mean value, 10 individuals (sec.)	Range (sec.)
*	58.9	31.0-120.0
30	—	40.0- ∞
45	—	50.0- ∞
60	—	70.0- ∞

* Immediately (zero-hr).

time of calcium, serum, and plasma. As seen from Table II the interaction time of calcium, serum, and plasma increased from a mean value of 9.4 seconds at zero-hour to 12.6, 14.3, and 15.8 seconds at 30, 45, and 60 minutes after coagulation, respectively. Following the intravenous injection of 50 mg of heparin the corresponding mean value increased to 23.7 seconds at zero-hour, 36.4 seconds at 30 minutes and 66.6 seconds at 45 minutes following coagulation. No mean value was obtained at 60 minutes following coagulation. One hour after intravenous administration of 75 mg of heparin the mean value of the interaction time of calcium, serum, and plasma increased to 58.9 seconds. It seems, however, of considerable significance that with this dosage the coagulum became exceedingly small, while the injection of 50 mg of heparin was devoid of such an effect. No mean values were obtained 30, 45, and 60 minutes following coagulation of blood obtained one hour after injection of 75 mg of heparin. C. *Interaction of fresh serum and fibrinogen solution.* Upon addition of fresh serum to a solution of bovine fibrinogen only a very minute and hardly visible coagulum was observed, the clotting time varying between 23 and 45 seconds in 6 experiments. D. *Interaction of serum and homologous* plasma. When fresh serum was added to an equal amount of homologous citrated plasma at zero-hour the mean clotting time was 9.0 seconds as obtained in 6 experiments, but rapidly lengthened to 43.5 seconds at 30 minutes, 60.3 seconds at 45 minutes and 70.0 seconds at 60 minutes following coagulation.

Discussion. The frequent failure of the Lee-White clotting time to reflect appropriately the action of heparin has been an incentive to search for other methods which may be applicable to its detection. A chemical assay for heparin in blood has been reported by Jaques(4). The fibrin appearance time of Weiner and Shapiro(5) may indicate a heparin effect provided a special apparatus with a rotating glass bulb is utilized. The citrate clotting time recently reported by the authors(6) indicates the anticoagulant effect of heparin with considerable sensitivity and does not require any extraneous agents besides a given suboptimal concentration of sodium citrate. While it appears that the citrate clotting time offers a more accurate indication of the anticoagulant action of heparin than the Lee-White clotting time, the procedure requires observation for prolonged periods of time.

The results of this study conclusively demonstrate that the coagulative power of fresh serum, as reflected in the very short prothrombin time at zero-hour, is substantially reduced by the therapeutic administration of heparin and in accordance with the dosage employed. It is of interest that the technic of the one-stage prothrombin time can be applied to the detection of the anticoagulant effect produced by heparin, not only within the first few minutes but long after its administration, provided serum and not plasma is used. It is obvious that this phenomenon is not adequately accounted for by the antiprothrombin action of heparin which is only of short duration and reflected in the plasma prothrombin time as reported by Laruelle(7) who demonstrated a prolongation of the one-stage prothrombin time varying with the concentration

of heparin per unit of plasma. Quick(8) similarly has shown that the prolongation of the prothrombin time is transient and most pronounced 5 minutes after the intravenous injection of heparin. It was, therefore, concluded that the prolongation of the serum prothrombin time, particularly at zero-hour as caused by heparin may be due to its effect either upon nascent thrombin, thromboplastin(9) or serum prothrombin conversion accelerator(10).

The assumption of free thrombin in fresh serum was not well supported by experiments in which serum at zero-hour was added to a fibrinogen solution. On the other hand, the solid coagulum obtained 9.0 seconds after the interaction of fresh serum and homologous plasma more strongly favored the assumption of the presence of free thrombin in fresh serum. The lengthening of this clotting time at later intervals pointed to a rapid neutralization of whatever amount of thrombin has been present after clotting. The instantaneous stabilization of the serum prothrombin time upon addition of sodium citrate also points to an extremely rapid neutralization of such thrombin content. On the other hand, the accelerating effect of calcium as shown in the interaction time of calcium, serum, and plasma (Table II) seems to be indicative of a definite and substantial thromboplastin effect of serum. In this procedure, serum, as an ingredient, assumes the role of thromboplastin as compared with the one-state Quick prothrombin determination.

The finding that bovine fibrinogen does not coagulate as readily as does homologous citrated plasma after addition of fresh serum, does not preclude the presence of thrombin. According to Laki(11), the clot formed in blood plasma is different from the clot formed from purified fibrinogen.

The prolongation of the interaction time of calcium, serum, and plasma following the administration of heparin appears to be due to both its antithrombic(1) as well as antithromboplastic actions(9). The assumption that it may be due to an effect upon serum prothrombin conversion accelerator (SPCA) seems to be difficult to reconcile with the finding that heparin added to serum plasma mixtures capable of lengthening the prothrombin time of plasma alone did not abolish SPCA activity (10).

Summary. 1. The one-stage prothrombin time of freshly coagulated and centrifuged blood is considerably prolonged after the intravenous administration of heparin. 2. The interaction time of calcium, serum, and plasma is similarly prolonged after intravenous injection of heparin. 3. The prolongation of the serum prothrombin time after administration of heparin is assumed to be due to both the antithromboplastic as well as antithrombic actions of the latter.

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Rate of Exchange of Sodium and Potassium Between Amniotic Fluid and Maternal System.* (21122)

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In a previous communication(1) we demonstrated that the mechanism of deuterium exchange between amniotic fluid and maternal circulation is that demanded by the theory of multicompartment systems, a general treatment of which has been presented by Sheppard and Householder(2). The exchange rate between the 2 compartments in 2 women at term was determined to be about 30 moles of water per hour, indicating a continuous formation, flow and reabsorption of the amniotic fluid.

There exists, in all probability, an electrolyte equilibrium between amniotic fluid and maternal plasma, which is analogous to that of lymph, cerebrospinal-, and extracellular fluid. The pathologic depletion or accumulation of water in these hypothetical compartments is associated with or conditioned by a disturbance in the electrolyte transfer, changes in hydrostatic pressure and the osmotic equilibrium. A fundamental step for further investigations on the etiology of these pathologic states would be a study of the rate of exchange of electrolytes, particularly that of sodium and potassium.

A comparison of these exchange rates could be used to evaluate the hypothesis that the amniotic fluid is not a transudate or formed by ultrafiltration of plasma, although the concentration of electrolytes in plasma and amniotic fluid is of the same order of magnitude. The present investigation was intended to give information on the quantity of sodium and potassium exchanged per hour and to demonstrate that sodium, potassium, and water exchange at their own characteristic rates.

The assumptions necessary for the application of tracers to the study of multicompartment systems are essentially the same as those previously cited(2). The expression for a 2-compartment system in a steady state

$$\log_e \left[\frac{a_1 - a_2}{a_{1(0)}}\right] = \rho t \left[\frac{1}{S_1} + \frac{1}{S_2}\right] = \frac{0.693\ t}{T_{½}}$$

is applicable to a multiplicity of tracers provided the designation of a and S is consistent(1). S_1 and S_2 refer to the amount of exchangeable species in compartments 1 and 2, and a_1 and a_2 to the specific activity of the tracer in these compartments at time t; $a_{1(0)}$ is the specific activity in compartment 1 at time zero. ρ is the exchange rate, the dimensions of which are determined by the units of t and S, and $T_{½}$ is the time necessary to replace ½ of the exchangeable species. If the logarithmic expression of the above equation is plotted against time, a straight line is obtained whose slope, the disappearance constant, is proportional to the exchange rate. If the amniotic fluid were formed by processes of filtration or diffusion, one would expect the slope of these lines for all constituents to be nearly equal though the exchange rates, expressed as moles per unit of time, may differ. In order to minimize the introduction of errors due to biologic variations, multiple simultaneous tracer experiments were carried out, using isotopes of hydrogen, sodium, and potassium. The tracers used in these experiments were chosen on the basis of the availability of selective analytic methods. Transabdominal puncture was performed on 2 pregnant volunteers at term who had been considered for caesarean section. The isotopes were administered into the amniotic fluid compartment by a method previously described(1,3). Samples of amniotic fluid and maternal blood were collected at predetermined intervals and the concentration of the tracers determined. Deuterium oxide was estimated by the falling drop method. Sodium-22, sodium-24, and potassium-42 were determined by their radio-

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activity. Because of the low dosage administered (3 μc) a Q-gas counter was used to determine the specific activity of the samples containing potassium-42. For the experiment where sodium-22 and sodium-24 were the only radio-tracers a well-type scintillation counter was found to be most advantageous.

The samples were counted immediately after collection and recounted after an interval of about 10 half life times to allow for the decay of sodium-24 or potassium-42. The remaining counts were due to sodium-22, which, when subtracted from the corrected total, permitted the estimation of potassium-42 or sodium-24 activity. The volume of the amniotic fluid was determined by the Congo Red method of Neslen *et al.*(3), and the total body water, total exchangeable sodium and total exchangeable potassium were estimated by extrapolation. The concentration of sodium and potassium in the amniotic fluid was determined by the flame-photometric technic.

Experimental. The first experiment was intended to test the correctness of the assumption that there exists a steady state which implies a constant amount of exchangeable species in both compartments and equal and opposite exchange rates. The experimental verification of this hypothesis is relatively simple for the exchange of sodium, because 2 easily detectable isotopes are available. Sodium-24 was injected into the maternal system and sodium-22 and deuterium oxide introduced into the amniotic fluid. The amount of radio-tracers injected into each compartment was proportional to the size of its pool (milliequivalents or moles of exchangeable sodium). In the determination of

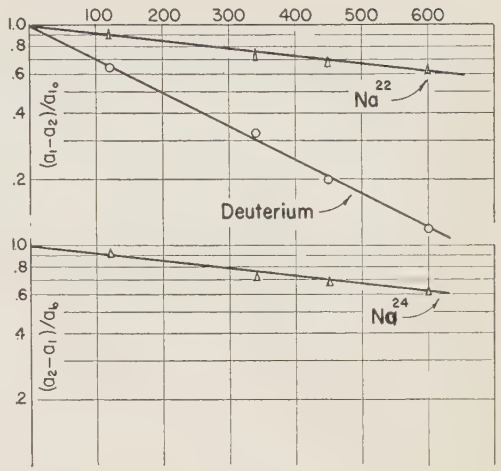

FIG. 1. Semi-log plot of function indicated on left against time in min. Upper cycle refers to transfer from amniotic fluid to mother; lower cycle designates reverse transfer.

TABLE I. Summary of Pertinent Data and Results Relating to Experiments Described in Text. Abbreviations "A.F." and "M.S." Refer to Amniotic Fluid and Maternal System, Respectively.

	Exp. I 30 cc D_2O and 3 μc Na^{22} inj. into A.F. 90 μc Na^{24} inj. into maternal circulation.	Exp. II 30 cc D_2O, 3 μc Na^{22} and 3 μc K^{42} inj. into A.F.
Disappearance constant in min.$^{-1}$ for		
Deuterium (A.F. → M.S.)	.00354	.014
Sodium (A.F. → M.S.)	.000907	.00273
Sodium (M.S. → A.F.)	.000822	—
Potassium (A.F. → M.S.)	—	.00284
Total body water (moles)	1680.0	1720.0
Vol of A.F. (")	126.0	39.0
Total exchangeable sodium (mEq)	2380.0	2460.0
Sodium concentration in A.F. (mEq/lt)	150.0	130.0
Total sodium in A.F. (mEq)	350.0	91.0
" exchangeable potassium (mEq)	—	2920.0
Potassium concentration in A.F. (mEq/lt)	—	3.4
Total potassium in A.F. (mEq/lt)	—	2.4
Exchange rate in moles/hr		
Water (A.F. → M.S.)	25.0	31.9
Sodium (A.F. → M.S.)	.016	.014
" (M.S. → A.F.)	.015	—
Potassium (A.F. → M.S.)	—	.00041

the transfer rate from amniotic fluid to maternal pool, the concentration of the tracer in the latter can be neglected while the determination of the opposite transfer rate necessitates a knowledge of the specific activity in both compartments as a function of time. Fig. 1 represents a semi-log plot of the function $(a_1\text{-}a_2)/a_{1(0)}$ against time for sodium-22, sodium-24, and deuterium oxide. The slopes of the lines for the 2 sodium tracers appear to be identical within the limits of error of the method, thus indicating that the transfer rates *in* and *out* of the amniotic fluid compartments are equal. By means of the other essential data listed in Table I the transfer rates were calculated as .016 and .015 mole per hour and 25.0 moles of water per hour.

In a second experiment deuterium oxide, sodium-22, and potassium-42 were injected into the amniotic sac. Since there was no detectable rise of radioactivity in the maternal serum, the value of a_2 was negligible for the 2 radiotracers. In the calculation of the disappearance constant for deuterium oxide, a rise in its concentration in maternal serum was taken into consideration. Fig. 2 represents a semi-log plot of the data obtained by the simultaneous application of deuterium, sodium-22, and potassium-42. The disappearance constants for the 3 tracers from amniotic fluid to maternal system were found to be .014 min.$^{-1}$ for water, .00273 min.$^{-1}$ for sodium and .00284 min.$^{-1}$ for potassium. Substitution of the data of Table I in the above equation permitted the calculation of the exchange rates as 31.9 moles of water per hour, 0.014 mole of sodium per hour and 0.00041 mole of potassium per hour.

Discussion. The transfer of sodium to the amniotic fluid has been investigated by Vosburgh, Flexner, Cowie, Hellman, Proctor, and Wilde(4). They reported that an average of about 6.9% of the sodium of the amniotic fluid is replaced per hour during gestational periods of 12 to 40 weeks. The transfer of water in another series of patients was found by these authors to be about 5 times faster.

There is an appreciable variation in the sodium and potassium concentration of the amniotic fluid which does not always parallel the electrolyte concentration of maternal

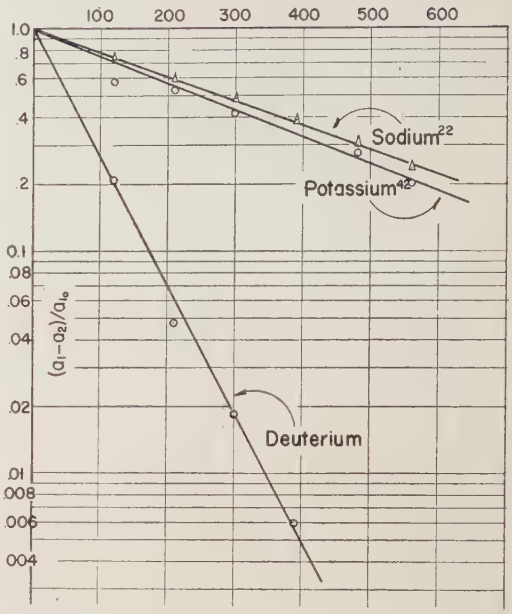

FIG. 2. Semi-log plot of function indicated on left against time in min. All tracers injected into amniotic sac.

serum. The application of average values for the sodium concentration of amniotic fluid and maternal serum may lead to errors, which, when added to the inherent errors in the determination of the compartmental size, would render the analysis worthless. For purposes of comparison the transfer of a species from one compartment to another should not be expressed as per cent of the total amount of the species within the compartment, but expressed as the rate of exchange, the dimensions of which are moles per unit of time. This incorporates variations in the amount of exchangeable species and its disappearance constant. The necessary prerequisite for the application of the theoretic considerations is a demonstration that the rates of exchange in both directions are identical.

The first of our experiments demonstrates that the exchange rates for sodium ion to and from the amniotic fluid are equal, within the experimental error of the methods. In the second experiment the transfer rates of deuterium, sodium, and potassium were determined. A comparison of the data for sodium of this and the preceding experiment shows that the transfer rates (14, 15 and 16 mEq./

hr) are of comparable magnitude while the disappearance constants differ by a factor of 3 (.00273 and .000907 min.$^{-1}$). The disappearance constant for potassium is roughly of the same magnitude as that of sodium, but a comparison of the exchange rates shows that the number of milliequivalents of sodium exchanged per minute is 35 times greater than the number of milliequivalents of potassium. The ratio of these exchange rates is about the same as the ratio of their respective concentrations in the amniotic fluid.

Summary. The exchange rate of sodium to and from the amniotic fluid was determined by the simultaneous application of sodium-22 and sodium-24. This exchange rate, in opposite directions, was found to be 15 and 16 mEq. of sodium per hour, respectively. The transfer rate of hydrogen, sodium, and potassium from the amniotic fluid to the maternal system was determined by the simultaneous application of tracers for each of these elements. The disappearance constants for sodium and potassium were found to be nearly equal, indicating that the same percentage of these elements is exchanged per unit of time, while that for deuterium was found to be 5 times greater. The exchange rates were calculated as 31.9 moles of water, .014 mole of sodium, and .00041 mole of potassium per hour. It is concluded that the water and electrolytes of the amniotic fluid are in dynamic equilibrium with maternal plasma, each exchanging at its own characteristic rate.

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Propagation of Salivary Gland Virus of the Mouse in Tissue Cultures.* (21123)

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The presence of greatly enlarged cells containing large intranuclear inclusions in the salivary glands of several species of rodents has been shown to be associated with a transmissible filterable virus(1-3). In each case the virus has proved to be entirely species-specific. In most species of animals basophilic cytoplasmic inclusions also occur in the cytoplasm of many of the enlarged cells, but in the experimental infections they appear later than the intranuclear inclusions(4). They are seen only occasionally in the salivary glands of infected mice. Intracellular inclusions resembling those occurring in the salivary glands of rodents have been observed in the salivary glands in 10 to 30% of autopsies of infants and young children(5-8) regardless of the cause of death and are present in other organs, as well as the salivary glands, in cases of inclusion disease of infancy. Transmission of the etiologic agent of the human disease to experimental animals has not been reported. The only reported attempt to propagate any of the salivary gland viruses of rodents in tissue cultures is that of Andrews(9). He tried to cultivate the salivary gland virus of the guinea pig in surviving tissue in a modified Maitland medium. Intranuclear inclusions appeared in mononuclear cells in the interstitial tissue in cultures of guinea pig testis and less constantly in cultures of guinea pig ovary, brain, or salivary glands. The results of all attempts to subculture the virus were negative. No inclusions were found in the

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subcultures, and inclusions did not occur in the salivary glands of young guinea pigs inoculated with the subcultures.

No adequate serologic tests for the identification of the salivary gland viruses have been developed. With the exception of 2 investigations dealing with the guinea pig virus there have been no serologic studies of any of the salivary gland viruses. Kuttner(1) reported that the sera of chronically infected guinea pigs did not prevent infection in guinea pigs inoculated with the serum-virus mixtures. Andrews(9), although he was unable to propagate the guinea pig virus in tissue cultures, showed that the sera of chronically infected guinea pigs was effective in preventing the production of the inclusions which occurred in cultures inoculated with the guinea pig virus. The large intranuclear inclusions occurring in greatly enlarged cells in the salivary glands of animals and young children are readily differentiated from the intranuclear inclusions occurring in other virus infections and have been considered as definitely diagnostic of such infections(1,3,10). The demonstration of these characteristic cytologic changes in the tissues has been the only means available for recognizing infection caused by any of the salivary gland viruses. Propagation of the salivary gland viruses in tissue cultures may facilitate the development of serologic tests for these viruses and offer a method for the study of the human salivary gland virus disease.

In the present study propagation of the murine salivary gland virus in cultures derived from mouse tissue was undertaken as a model for future attempts to isolate the human virus. This report is concerned with the isolation and propagation of the mouse salivary gland virus.

Material and methods. Tissue cultures. Mouse embryonic tissue was cultured in roller tubes (20 x 100 mm). Mouse embryos were obtained during the third week of gestation. The head was discarded and the remainder of the embryo was minced with scissors. Fifteen to 20 fragments were embedded in a thin layer of clotted chicken plasma on the lower two-thirds of the roller tubes. The nutrient media employed consisted of Hanks' balanced salt solution and ox serum ultrafiltrate in the proportion of 3:1, chick embryonic extract (10%) and human ascitic fluid (5%). Fifty units of penicillin and 50 μg of streptomycin per ml of media were added. The cultures were incubated at 35°C, and the nutrient fluids were changed at intervals of 2 or 3 days. The cultures were usually maintained for 8 to 14 days before the virus was inoculated. At this time the cultures consisted largely of spindle cells, presumably fibroblasts. A small amount of epithelial-like growth persisted in some cultures. For histological examination the cultures were fixed in the tubes with Bouin's solution and stained in the tubes with hematoxylin and eosin. In a few instances fragments of the cultures were removed from the tubes before fixation and placed in Bouin's solution. After fixation a number of fragments were embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin.

Origin of virus and inoculation of cultures. The mice used in these experiments were from a colony in which the latent salivary gland virus does not seem to be present. This colony was isolated from other mice and will be referred to as the uninfected colony. Several adult mice obtained from the Wernse Laboratory of Cancer Research at Washington University were shown to have the salivary gland virus infection as indicated by the demonstration of small numbers of the characteristic large intranuclear inclusions in cells of the salivary glands. Portions of the salivary glands of 3 of these mice in which inclusions were demonstrated were pooled and used to pass the virus to young adult mice of the uninfected colony. Many of the characteristic intranuclear inclusions were demonstrated in sections of the salivary glands of passage mice killed 2 weeks following the subcutaneous inoculation of 0.2 ml of a 10% suspension of the salivary gland material. The virus has been maintained in young adult mice from the uninfected colony by serial subcutaneous passages with salivary gland material at intervals of 2 to 3 weeks over a period of several years.

Salivary gland material from mice infected 2 weeks previously by subcutaneous inocula-

Fig. 1

Fig. 2

FIG. 1. Control uninoculated culture of mouse embryonic tissue. Fixed in culture tube, hematoxylin-eosin stain. × 90.

FIG. 2. Culture of mouse embryonic tissue 9 days following inoculation with mouse salivary gland virus. Fixed in culture tube, hematoxylin-eosin stain. × 90.

tion of the virus was used for the inoculation of the tissue cultures. Recently infected mice were used as the source of virus in order to have material of a relatively high viral titer. The salivary glands of 3 mice were ground and diluted 1:10 by weight in Hanks' solution. After centrifugation the supernatant fluid was further diluted 1:10 and a portion was filtered by means of the Swinny adaptor. For inoculation of the tissue cultures the filtrate was added to fluid culture media in the desired dilutions and 2 ml of the inoculated media placed on each culture when a change of nutrient fluid was made. Usually the final dilution of the infective salivary gland material in the inoculated culture media was 10^{-4}.

Titration of virus. The salivary gland material from recently infected animals was often infective for mice 3 to 5 weeks of age in dilution as great as 10^{-8}. Therefore the filtered infective salivary gland material used as the inoculum for cultures was titrated in mice in 10-fold dilutions to 10^{-9} and the pooled nutrient fluids removed from cultures after periods of incubation were titrated in 10-fold dilutions to 10^{-7}. Three mice, 3 to 5 weeks of age, from the uninfected colony were inoculated intraperitoneally with 0.2 ml amounts of each serial 10-fold dilution. The microscopic demonstration of the characteristic intranuclear inclusions in the salivary glands of these mice when killed 2 weeks following intraperitoneal inoculation was used as the criterion for infection with the virus. Three to 5 sections from each animal cut to include as great an area of salivary gland tissue as possible were examined. The salivary glands of 3 to 5 uninoculated control mice were examined for each titration. The greatest dilution of the infective material which produced inclusions in the salivary glands of 2 of the 3 test mice was considered as the end point of the titration. This method was used in preference to one dependent upon death of one-week-old mice, because the smallest amount of virus which will uniformly cause the death of one-

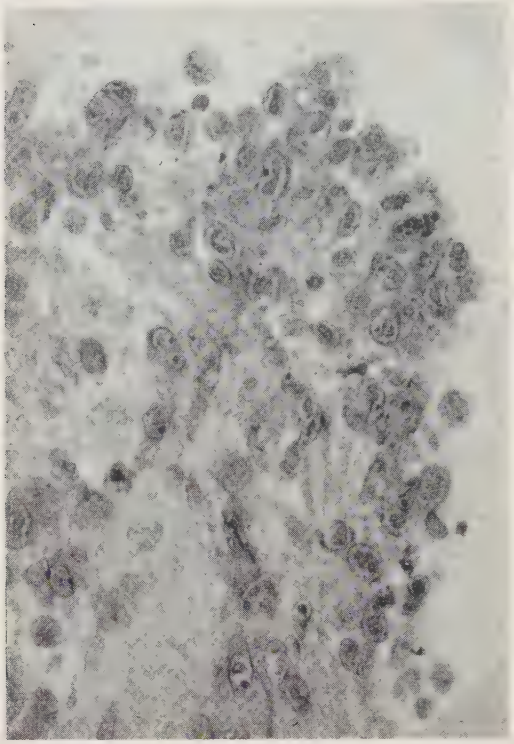

FIG. 3. Section of fragment from culture inoculated with mouse salivary gland virus. Large intranuclear inclusions are shown. Hematoxylin-eosin stain. × 400.

week-old mice within 2 weeks is considerably greater than the infective dose of virus for mice 3 to 5 weeks of age as indicated by the occurrence of inclusions in the salivary glands.

Experimental results. Cytologic changes occurred in tissue cultures when virus was added to the cultures by replacement of the nutrient media with 2.0 ml of media containing the infective salivary gland material in dilution of 10^{-4}. In 3 experiments when the infective material in this dilution was added to 3 or 4 culture tubes focal cytologic lesions occurred in all tubes on the 6th or 7th day. Small groups of cells, which were large, rounded and refractile, contrasted sharply with the surrounding spindle cells. In the next few days the focal lesions increased in number and enlarged by the involvement of adjacent cells in the process. Within 3 to 5 days almost every cell in the culture became affected (Fig. 1 and 2). Some cells began to degenerate within 2 or 3 days after the lesions appeared, but a few of the large refractile cells persisted into the 4th week after inoculation of the cultures.

Such changes never appeared in control uninoculated cultures nor in cultures inoculated with materials prepared from salivary glands of mice from the uninfected colony, which did not show inclusions when examined microscopically.

In cultures that were fixed and stained in the tubes 9 days after inoculation a large intranuclear inclusion was observed in almost every intact large cell and an occasional cell contained 2 or 3 nuclei each having an inclusion. The intranuclear inclusions tended to be somewhat basophilic when stained with hematoxylin and eosin. The inclusion was separated from the nuclear membrane and the shape of the inclusion frequently corresponded closely to that of the nucleus (Fig. 3). In the sections of the fragments which had been embedded in paraffin the intranuclear inclusions appeared granular or, in some instances, consisted of coarse clumps of material. One or 2 small masses of chromatin material staining more deeply basophilic than the inclusions were present in most nuclei, usually attached to the nuclear membrane.

In 2 serial passages of the virus from cultures inoculated with salivary gland material, cytologic lesions appeared in all the subcultures and were of the same character as those observed in the original cultures.

TABLE I. Multiplication of Mouse Salivary Gland Virus in Tissue Cultures.

Day of incubation when nutrient fluid was titrated	No. of nutrient fluid changes before titration of fluid	Infective titer of pooled fluid from cultures*	Dilution of original sal. gl. material before fluids were titrated
2	0	10^{1}	10^{-4}
5	1	10^{3}	10^{-5}
7	2	10^{4}	10^{-6}
9	3	10^{4}	10^{-7}
12	4	10^{5}	10^{-8}
16	6	10^{3}	10^{-10}
19	7	10^{4}	10^{-11}

The estimated increase of virus after 19 days was in the order of 10^{9} times.

* Salivary gland material having an infective titer of 10^{6} was diluted to 10^{-4} in the nutrient fluid when the infection was initiated.

TABLE II. Multiplication of Mouse Salivary Gland Virus in Serial Tissue Cultures.

Passage	Days of incubation subcultures made		No. of nutrient fluid changes		Mouse infective titre of pooled fluid, end of incubation period		Dilution of original sal. gl. material, end of incubation period	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1*	Exp. 2†	Exp. 1	Exp. 2
I	7	14	1	5	10^{5}	10^{5}	10^{-5}	10^{-11}
II	6	9	1	3	10^{6}	10^{5}	10^{-8}	10^{-19}
III	7	12	1	4	10^{5}	10^{4}	10^{-11}	10^{-28}

Estimated increase of virus was in the order of 10^{8} times in the 1st exp.; 10^{20} times in the 2nd exp.

* Salivary gland material having an infective titre of 10^{8} was diluted to 10^{-4} in nutrient fluid when infection was initiated. Nutrient fluid placed on subcultures when they were inoculated was a dilution of 1:100 of pooled fluids from preceding cultures.

† Salivary gland material having an infective titre of 10^{6} was diluted to 10^{-6} in nutrient fluid when infection was initiated. Nutrient fluid placed on subcultures when they were inoculated represented a dilution 10^{-5} of pooled fluids from preceding cultures.

Multiplication of virus. The salivary gland virus disease as characterized by the large intranuclear inclusions in enlarged cells of the salivary glands was reproduced in every instance in mice inoculated subcutaneously or intraperitoneally with the supernatant fluids withdrawn from original cultures or subcultures showing the cytologic changes. To demonstrate the extent of multiplication of the virus in tissue cultures, the infective titers of pooled supernatant fluids removed at intervals from one set of 3 cultures inoculated with the infective salivary gland material were determined by titrations in mice and compared with the infective titer of the inoculum. Considering each successive removal and replacement of the nutrient fluid as a further 10-fold dilution of the original inoculum, it is estimated that an increase in virus in the order of 10^{9} times occurred during a 19-day period (Table I).

The extent of multiplication of the virus during 3 serial passages was also determined. Pooled fluids from 2 or 3 culture tubes were titrated in mice when the subcultures were made and in the case of the second subcultures at the end of the period for which the cultures were maintained. The results of the titrations together with the calculated dilution of the original inoculum during the course of the experiment indicated that the increase in virus during 3 serial passages over a period of 20 days was in the order of 10^{8} times (Table II).

In a second experiment less virus was inoculated into both the original cultures and the subcultures in order to demonstrate multiplication of the virus more clearly. When the dilution of the infective salivary gland material effected during the experiment is calculated, it is estimated that the increase of virus over a period of 35 days during which 2 serial subcultures were made was in the order of 10^{26} times (Table II).

Summary and conclusions. Cytologic changes including large intranuclear inclusions were produced in cultures of mouse tissue inoculated with salivary gland material from mice infected with the salivary gland virus. They also occurred in 2 serial subcultures. The large intranuclear inclusions resembled those occurring in cells of the mouse salivary gland. Further evidence that the mouse salivary gland virus has been propagated in serial passages in cultures derived from mouse embryonic tissue is as follows. After 3 serial passages of the infective agent in the cultures the salivary gland virus disease, as characterized by specific intranuclear inclusions, has been reproduced in the salivary glands of mice by intraperitoneal inoculation of the supernatant fluids from the cultures. Also the infective titers of supernatant fluids withdrawn from cultures at intervals have been determined by titrations in mice. The results of the titrations, together with the calculated dilutions of the original inocula effected during the course of the experiments, demonstrated

significant increase in the virus in 3 serial passages in tissue culture.

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Uric Acid in Two Patients with Wilson's Disease (Hepatolenticular Degeneration).* (21124)

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(Introduced by Fred R. Griffith, Jr.)
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In the course of a complete laboratory examination of a brother and sister both suffering from Wilson's disease, it was discovered that the serum urate levels in both individuals were unusually low (1-2 mg %), according to the colorimetric method(1). To elucidate the mechanism of this behavior it was decided to determine uric acid pool size and turnover rate by injecting N^{15} uric acid in the manner previously described(2).

The male subject was injected intravenously with 21.7 mg of N^{15} uric acid (14.81 atom % excess), and all urine was collected *ad libitum* for 5 days. From these results the pool size was calculated to be 442 mg and the turnover rate, 1.95 pools per day. The mean excretion for 4 days, determined by the isotope dilution method in the period just after the other measurements was 848 mg per day. The turnover was 442 x 1.95 = 862 mg and the per cent of the turnover excreted was 848/862 x 100 = 98%(4).

A similar study was performed on the female subject, using 19.5 mg of N^{15} uric acid. The pool size was calculated to be 304 mg and the turnover rate was 1.78 pools per day. The turnover was therefore 541 mg. The mean excretion for 8 days immediately following the test period was 391 mg and the per cent of the turnover that was excreted was 72%.

To test the possibility that the kidney tubules were completely inhibited in so far as their resorption of uric acid was concerned, the drug, Benemid, [p(di-n-propylsulfamyl) benzoic acid] was administered to the female subject and the uric acid injection was repeated. Sirota *et al.*(3) have demonstrated that this drug selectively inhibits tubular resorption of uric acid. The subject was given 3 × 0.5 g Benemid per day by mouth for 6 days and was then injected intravenously with 20.8 mg of isotopic uric acid. The pool size was determined to be 167 mg and the turnover rate 2.34 pools per day. The turnover was therefore 391 mg and the per cent turnover excreted was 86%, based on a mean excretion of 452 mg during 12 days on Benemid. The mean urinary excretion after Benemid was not statistically different from the control value, the P value for the t test being well above the 10% level.

After Benemid therapy was started on the female subject, her clinical condition became considerably worse. After cessation of the therapy she had not improved. Her present serum uric acid level is approximately 1 mg

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TABLE I. Effects of Feeding 2 × 2 g of Commercial Ribonucleic Acid (RNA) on Uric Acid (UA) Levels in a Patient with Wilson's Disease.

	Serum UA, mg %	Urinary UA, mols N/day	Extra UA, % of ingested purine	Non-UA purines, mols N/day
Before RNA	1.3 to 1.6	20.6		8.9‡
After 9 days RNA feeding	3.0			
" 16 " " "	1.8			
During RNA feeding (Wilson's disease)		34.1*	55	9.9
Idem (normal controls) †			61, 73	
" (gouty patient) †			47	

* Statistically significant at 0.1% level.
† See reference(5). N^{15}-nucleic acid fed in one dose.
‡ " " (6).

%. Because of the coincidence of relapse and therapy, the same regimen was not instituted in the male subject.

To test the hypothesis that the neurological symptoms of the disease might be related to the low serum uric acid level, nucleic acid was fed with the results shown in Table I.

Discussion. The pool size of both the individuals with Wilson's disease was markedly below normal and the turnover rate was approximately twice the normal value. This would be consistent with an inhibition of tubular rate resorption, especially in the male where 98% of the uric acid turned over was excreted via the kidney. The female, on the other hand, had a normal per cent of turnover excreted and was susceptible to Benemid action insofar as the pool size and turnover rate were concerned. That Benemid did not cause a significant increase in urate excretion is in agreement with other studies from this laboratory(7) which indicate that unless there are increased serum urate levels, Benemid seems to have no uricosuric effect.

It has been reported(8) that the blood amino acid level in Wilson's disease is normal even though an aminoaciduria is present. In contrast to this behavior, the serum uric acid levels and the uric acid pool size are decreased, according to the data presented here.

Several mechanisms could be suggested to account for the difference in behavior of these 2 types of substances but more data are needed before any explanation could be validated. It is apparent that any relationship between uric acid and the etiology or pathogenesis of Wilson's disease is purely speculative on the basis of these studies, but the findings are interesting nevertheless.

Summary. A brother and sister with Wilson's disease (hepatolenticular degeneration) were found to have low serum urate levels and were injected with isotopic uric acid for pool size and turnover rate determination. The pool size of both was well below normal and the turnover rate was approximately twice the normal value. Benemid accentuated these findings in the female but was not tried in the male because of its adverse clinical effect. The rapid rate of removal of uric acid is compared to the phenomenon of aminoaciduria that is common in this disease. When ribose nucleic acid was fed to the male subject there was a statistically significant increase in urinary uric acid and this "extra" uric acid was equivalent to about 55% of the calculated purine ingested. There was no significant rise in the excretion of non-uric acid purines after nucleic acid feeding and this suggests that exogenous purine is mainly converted to uric acid before excretion.

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Bromsulfalein Retention Resulting from Liver Damage by a Carbon-Free Filtrate of India Ink.* (21125)

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India ink has been widely used to block the reticuloendothelial system(2). Since bromsulfalein clearance was impaired by India ink in a manner roughly proportional to the dose of ink, Mills and Dragstedt(3) proposed the use of the BSP clearance test as a measure of reticuloendothelial blockade. The interpretation of these experiments was challenged by Brauer and Pessotti(4) who suggested on the basis of *in vitro* experiments that the primary effect of India ink on the liver consisted of circulatory impairment. Subsequent work in our laboratory(5) suggested that the effect of India ink on liver function was neither the result of reticuloendothelial blockade nor of circulatory impairment. Apparently the observed results were not produced by the carbon of this preparation. Consequently, we inferred the presence of some toxic component in the aqueous phase of the India ink. The idea that India ink contains a soluble liver poison has been suggested by Victor *et al.*(6) on the basis of preliminary experiments with vital stains. The present investigation offers additional evidence that the action of India ink on BSP excretion is the result of the toxicity of the dispersing medium rather than the blocking effects of particulate matter.

Methods. India ink filtrate was prepared by repeatedly passing 8% Higgins India ink in 0.9% saline (v/v) through a Seitz filter under pressure, until the filtrate was water clear to the eye. The filtrate appeared slightly yellow and its pH was equal to that of the original ink. We observed initially that when ink was filtered by employing suction instead of pressure the pH of the filtrate was less than that of the ink and this method of preparation was discarded. The carbon suspension was prepared as follows: 400 mg of Cabot Elf-O carbon black was suspended in 50 ml of water which was agitated in an ultrasonic generator‡ for 5 minutes subsequent to the addition of carbon. Five ml of 8% gelatin were added to stabilize the colloid and this was followed by 0.9 g NaCl. The resulting mixture was diluted to 100 ml with water. The carbon suspension thus produced contained approximately the same amount of carbon as 8 ml of undiluted India ink and the

TABLE I. BSP Concentration at Various Intervals before and after Carbon Administration.

	BSP concentration (mg %)										
Dog wt, kg	Min. after 1st dose of BSP				Min. after carbon administration			Min. after 2nd dose of BSP			
	2	4	6	30	10	20	30	2	4	6	30
9.3	4.83	2.76	2.04	.94	.89	.55	.43	4.78*	3.60*	2.99*	1.02
19.5	4.44	2.44	1.40	.41	.30	.23	.20‡	4.33*	2.82*	1.89*	.67
10.7	7.86	5.11	4.54	2.55	2.23	2.00	1.84	7.95	6.59	5.61	3.76
11.1	5.42	3.18	2.05	1.94	1.76	1.68	1.54	9.34	7.14	5.69	3.17
6.8	6.83	4.38	3.22	2.60	2.38	2.19	2.03	7.46	5.10	3.70	3.36
Avg	5.88	3.57	2.65	1.69	1.51	1.33	1.21	7.16	5.28	4.11	2.40
S.E.†	.64	.51	.55	.44	.40	.40	.38	.73	.70	.67	.64

* These values are 3, 5 and 7 min. after BSP instead of 2, 4 and 6 min. Averages of these columns are calculated from the estimated 2, 4 and 6 min. values for these 2 dogs.

† Stand. error.

‡ Estimated from the 20 min. value.

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† Predoctoral Fellow of the Life Insurance Medical Research Fund.

‡ General Electric Catalogue No. 8665966g3, 300 kc.

TABLE II.
BSP Concentration at Various Intervals before and after India Ink Filtrate Administration.

Dog wt, kg	BSP concentration (mg %) — Min. after 1st dose of BSP 2	4	6	30	Min. after filtrate administration 10	20	30	% of inj. dose released*	Min. after 2nd dose of BSP 2	4	6	30
7.3	6.21	3.60	2.45	.84	2.59	2.77	2.55	17.1	12.2	11.7	10.9	8.22
18.0	6.16	3.66	2.36	.52	1.71	2.02	2.10	15.8	14.5	12.5	12.2	7.38
8.2	4.70	3.28	1.51	.12	.56	.62	1.22	11.0	10.2	8.86	7.45	2.83
9.5	7.59	4.25	2.68	1.16	2.39	2.82	2.92	17.6	14.3	12.8	12.2	7.60
11.3	6.44	3.08	1.34	.60	1.95	2.92	2.26	16.6	13.3	12.6	11.5	7.25
Avg	6.22	3.57	2.07	.65	1.84	2.23	2.21	15.6	12.9	11.7	10.9	6.66
S.E.†	.46	.20	.27	.17	.36	.43	.28	1.2	.79	.72	.88	.92

* Calculated on the basis of a 5% plasma volume.
† Stand. error.

microscopic appearance of this suspension was quite similar to that of the 8% India ink. The dogs used in the experiments were anesthetized with Na pentobarbital. Ink filtrate, carbon emulsion or BSP were injected into the femoral vein of one leg and blood samples were obtained from the opposite leg. The blood BSP concentration was determined by the method of Gaebler(7).

Results and discussion. The 5 dogs in Table I were injected with BSP (5 mg/kg) and their plasma levels were determined 2, 4, 6, and 30 minutes after BSP injection (columns 2-5). At the 30-minute interval 100 cc of carbon suspension was given intravenously over a period of 4-5 minutes. This carbon disappeared rapidly from the blood stream and most of it appeared to be in the liver when the animal was autopsied. The center 3 columns of Table I give the plasma BSP levels 10, 20, and 30 minutes after the end of the carbon injection. In all animals the BSP levels continued to fall throughout this period. The last 4 columns of Table I present the plasma BSP levels after a second dose of BSP which was administered immediately following the last 30-minute blood sample. The previous administration of carbon obviously had little or no effect on the rate at which BSP was removed from the circulation.

Table II gives the data for similar experiments except that India ink filtrate was injected instead of the carbon suspension. Two striking differences between these and the above results are apparent: 1) during the 30-minute period following the administration of filtrate 15.6% of the injected BSP was released into the circulation and the BSP concentration in the blood increased instead of showing a decrease; 2) the removal of the second dose of BSP was markedly impaired after the dose of ink filtrate. Thus, it appears that the carbon-free India ink filtrate produced the same effects as were previously obtained with whole India ink(5).

Our observation(5) that a dose of India ink which markedly inhibits BSP excretion has no measurable effect on the rate of removal of colloidal gold from the bloodstream throws considerable doubt on the assumption (3) that doses of India ink which interfere with dye excretion block the Kupffer cells of the liver. The demonstration that a carbon-free filtrate of India ink inhibits BSP excretion whereas carbon in a gelatin stabilized suspension has no inhibitory effect on BSP clearance is further evidence that some factor other than the carbon particles of India ink is toxic to the hepatic excretory system.

Victor *et al.*(6) observed that a combined alcoholic extract and acid supernatant of India ink interfered with the excretion of brilliant vital red into the biliary system. These authors suggested that some component of India ink might interfere with the transfer of dye from the liver phagocytes to the bile. However, their hypothesis does not explain the fact that both India ink and India ink filtrate caused the release of BSP already stored in the liver. If India ink merely blocked the transfer of BSP from liver cells to the bile canaliculi, one would not expect such a release of dye. However, the liberation

of BSP from the liver by India ink would appear plausible if India ink contained some substance which diminished the BSP-concentrating ability of the liver cells.

Our observations that the effect of India ink on BSP clearance is not produced by gelatin stabilized carbon particles and that doses of India ink sufficient to inhibit BSP excretion do not influence the phagocytic process(5) indicate that BSP clearance is not a suitable index of Kupffer cell activity.

Summary. The administration of a gelatin-stabilized carbon suspension did not affect bromsulfalein (BSP) clearance in dogs. However, a carbon-free filtrate of Higgins India ink markedly inhibited BSP uptake and effected the release of previously stored BSP from the liver. It appears that the effect of India ink on BSP excretion is not due to reticuloendothelial blockade but rather to a toxic effect of some soluble component of the ink on the liver. It is suggested that in dogs BSP clearance is not a suitable index of Kupffer cell activity.

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Effect of Quinidine on Papillary Muscle.* (21126)

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The effect of quinidine on the excitability of heart muscle has been studied in experimental animals as well as in man(1). The inhibition by quinidine is manifested by a depression of contraction and an increased refractory period. Although evidence is scanty it is generally believed that the effect of quinidine on the heart is due to a slowing of recovery processes resulting from impaired metabolism. In the present work simultaneous changes in contraction and oxygen uptake of the isolated cat papillary muscle following the administration of quinidine were observed. The results were further analyzed in the light of the effects of quinidine on the metabolism of heart slices.

Method. The method described previously (2) was used for the simultaneous observation of contraction and oxygen consumption of the muscle. The oxygen consumption and anaerobic glycolysis of cat heart slices were determined manometrically by a conventional Warburg method. The thickness of the slices

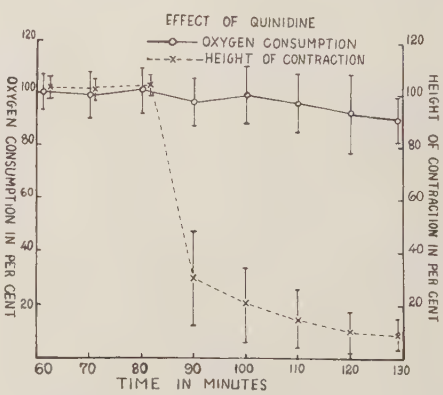

FIG. 1. Effect of quinidine (1:10000) on oxygen consumption and height of contraction of papillary muscles (7 exp.). In this and following figures, the 99% confidence limits of each mean are presented graphically by the vertical lines.

* This investigation was supported by research grant from the National Heart Institute of the National Institutes of Health, Department of Health, Education and Welfare.

was about 0.3 mm. The mediums used in the experiments for measurement of oxygen consumption and anaerobic glycolysis were Krebs-Ringer-phosphate and Krebs-Ringer-bicarbonate, respectively(3). Papillary muscles were prepared from the right ventricle of the cat heart according to the method of Cattell and Gold(4). Quinidine sulfate, U.S.P. (Merck) was used in these experiments.

Results. Effect of quinidine on the oxygen consumption and contraction of heart muscle. Muscles were stimulated at a rate of one per second throughout experiment. Results are summarized in Fig. 1. At the arrow, quinidine was added to make a final concentration of 1:10000. Immediately following addition of the drug, a rapid decrease in the height of contraction occurred while there was no significant change in oxygen consumption.

To compare this decline in contraction with that produced by anoxia, the system was equilibrated with an atmosphere of 95% nitrogen and 5% carbon dioxide for about 30 minutes. Muscles from oxygenated Krebs-Ringer-phosphate solution were then suddenly introduced into the anaerobic system and contractions recorded during stimulation at a rate of one per second. Results are shown in Fig. 2. The decrease in height of contraction following quinidine was included in the figure for comparison. Under an anaerobic condition, height of contraction of the muscle declined rapidly, reaching about 10% of original level in 20 minutes. Deterioration of muscular contractions induced by anoxia was thus simi-

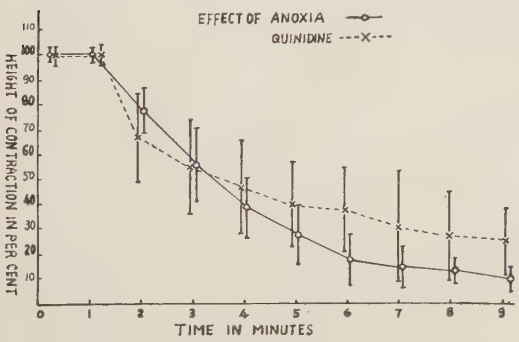

FIG. 2. Effects of anoxia and quinidine (1:10000) on height of contraction of papillary muscles (8 exp.).

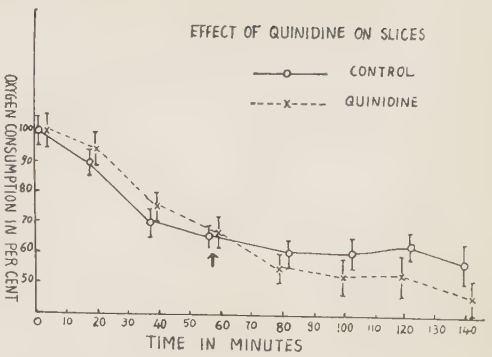

FIG. 3. Effect of quinidine (1:10000) on oxygen consumption of heart slices (12 exp.).

lar to that produced by 1:10000 quinidine both in rapidity and in intensity.

Effects of quinidine on the oxygen consumption and anaerobic glycolysis of heart slices. Fig. 3 represents results obtained in experiments on oxygen consumption. In comparison to control tests there was a little less oxygen consumption following addition of quinidine (1:10000) but the magnitude of this difference was not statistically significant. The effect of the drug on anaerobic glycolysis of slices is shown in Fig. 4. Here again, there appeared to be slight inhibition of glycolysis following addition of the drug but this was not statistically significant.

Discussion. Decrease in oxygen consumption and in glycolysis of heart slices treated with quinidine have been previously reported (5,6). In the present experiments with heart slices, decreases in these 2 functions appeared to follow administration of 1:10000 quinidine, but these effects were of doubtful significance. Quinidine in this concentration exerted no significant effect on oxygen consumption of stimulated papillary muscles. On the other hand, 1:10000 quinidine had a profoundly depressant effect on contraction of stimulated muscle which almost equalled the effect of complete anoxia in its intensity and rapidity.

The effect of complete blockade of glycolysis by iodoacetate on contractility of papillary muscle is very much slower than that produced by complete anoxia(7). These data suggest that inhibition of glycolysis or oxygen uptake *per se* by quinidine, does not satisfactorily explain the profound depressant action of the drug on contraction. This interpreta-

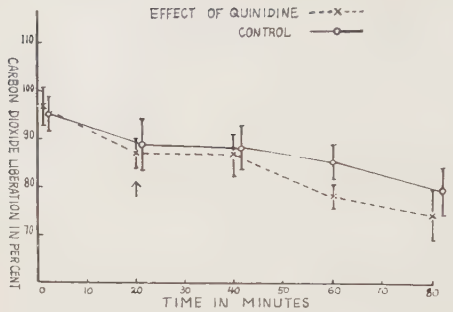

FIG. 4. Effect of quinidine (1,10000) on anaerobic glycolysis of heart slices (9 exp.).

tion does not exclude the possibility that quinidine may specifically inhibit the metabolic system required to maintain the functional integrity of the cellular membrane. This possibility, however, implies the assumption that the cellular membrane and cell itself derive their energy from different sources.

Conclusion. 1. Quinidine was found to have a profound inhibitory effect on contractility but only a slight effect on oxygen uptake of contracting cat papillary muscle. 2. Oxygen consumption and anaerobic glycolysis of the heart slices were not significantly inhibited by quinidine.

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Effect of Adrenalectomy on Wound Healing in Normal and in Stressed Rats.* (21127)

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That non-specific stress of sufficient magnitude results in depression of the bursting pressure of standard laparotomy incisions in rats has been reported by the authors(1). In each case stress, associated with depression of healing, was observed to have resulted in some degree of adrenal hypertrophy. It was postulated that the stress-induced inhibition of healing might be due to an accelerated rate of endogenous adrenal cortical secretion, sufficient in magnitude to inhibit the healing process. The hypothesis is compatible with the fact that ACTH itself inhibits the rate of healing(1-3), presumably due to increased cortico-adrenal secretion.

In order to test this hypothesis, healing was studied in adrenalectomized rats. Laparotomy wounds, made 15 days after bilateral adrenalectomy, showed bursting pressures equal to or greater than those of normal control rats. When stress was applied to adrenalectomized animals, the mortality rate became prohibitive. Therefore, the effect of stress upon healing was studied in adrenalectomized rats, which were given a small fixed daily maintenance dosage of adrenal cortex extract.

Methods. Young adult male rats of the Wistar strain, weighing about 175 g were used. Bilateral adrenalectomy was performed under ether anesthesia by the posterior approach. In sham-operated animals the operation was identical except that the adrenal glands were not removed. The animals were maintained in individual cages on Purina Laboratory Chow. After adrenalectomy, a 1% solution of sodium chloride was substituted for drink-

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TABLE I.
Effect of Adrenalectomy on Mean Bursting Pressure of Fifth Day Laparotomy Wounds.

	Days post-laparotomy			
	4	5	7*	10
Normal control	49 ± 2.3 (10)†	75.4 ± 2.2 (27)	108.9 ± .6 (19)	122.7 ± 2.6 (28)
Sham-adrenalectomy		71.8 ± .8 (12)		
Adrenalectomy (15 days pre-laparotomy)	52.3 ± 1.0 (10)	82.1 ± 3.2 (18)	118.9 ± 2.9 (15)	121.1 ± 4.4 (14)

* Difference in bursting pressures between the normal control and the adrenalectomized rats was significant only on the 7th day post-laparotomy ($P < .01$).
† Figures in parentheses indicate the No. of animals in each experiment.

ing water. Environmental temperature was 75 ± 5°F.

Laparotomy incision was made according to a standard technic(1) 15 days after adrenalectomy. The bursting pressure of the incision was tested 4, 5, 7, and 10 days postoperatively in groups of 10 or more animals, respectively, as previously described(1). These results were compared with similar incisions in normal control animals and in some cases with sham-adrenalectomized control rats. The stress employed consisted of excision of a patch of skin measuring 6 x 3 cm from the backs of the animals under ether anesthesia. No dressing was applied. This was done 4 days prior to laparotomy. Injections of Beef Adrenal Cortex Extract Aqueous (Upjohn)† were begun immediately prior to initiation of stress in a series of adrenalectomized rats. A dosage of 2 ml twice daily was injected subcutaneously. This is equivalent to 0.8 glycogenic unit. It is estimated to constitute an average maintenance dose for the rat(4). In one experiment desoxycorticosterone in sesame oil (5 mg daily, subcutaneously) was substituted for adrenal cortex extract. In order to rule out the possibility that these hormones themselves might affect the rate of healing, the effect of the same doses of cortex extract and of DOCA was tested in groups of stressed nonadrenalectomized animals.

Results. Table I summarizes the effect of adrenalectomy upon the rate of healing, when compared with normal control rats. On the 4th and the 10th postoperative days, there is essentially no difference between the adrenalectomized and the normal rats. On the 5th day the mean bursting pressure is increased in the adrenalectomized animals, but this increase is not of statistical significance (P .05). On the 7th postoperative day, however, the adrenalectomized group had a significantly greater mean bursting pressure than did the normal controls ($P < .01$).

From Table II it can be seen that skin-excision-stress resulted in significant depression of the mean bursting pressure. Treatment with either cortex extract or DOCA did not improve this mean bursting pressure. When the same stress was applied to adrenalectomized rats, maintained on cortex extract, there was no depression of the mean bursting pressure. These wounds are significantly stronger than those of stressed, nonadrenalectomized rats. Similar results are evident following the use of DOCA for supportive therapy, except that here the series is not large enough for statistical significance.

Discussion. Skin-excision-stress in the adrenalectomized rat, supported by small doses of cortex extract, failed to depress the mean bursting pressure of the 5th day laparotomy wound. In the normal control rat, stress did result in depressed bursting pressure in both untreated rats and in animals receiving the same dosage of cortex extract. It appears, therefore, that under the conditions of this experiment, stress-induced inhibition of healing may be attributed to an accelerated rate of cortico-adrenal secretion following stress.

If the impairment of healing following stress is due to an accelerated rate of cortical

† We are indebted to Dr. W. T. Haines of Upjohn Co. for a generous supply of this product.

TABLE II. Effect of Stress in Adrenalectomized Animals.

	No. rats	Mean bursting pressure	n	t	P
Normal control	27	75.4 ± 2.2	—	—	—
1. Skin-excision-stress	34	60.0 ± 1.5	—	—	—
2. Skin-excision-stress + A.C.E. 4d pre-lap.	8	56.8 ± 2.3	40	—	N.S.*
3. Adrenalectomy 15d pre-lap., skin-exc. and A.C.E. 4d pre-lap.	12	74.0 ± 2.1	44	3.46	<.01
4. Skin-exc.-stress and DOCA 4d pre-lap.	12	53.8 ± 2.9	44	—	N.S.*
5. Adrenalectomy 15d pre-lap., skin-exc. and DOCA 4d pre-lap.	5	69.1 ± 1.3	37	3.33	.037*

* N.S. = Not significant as compared with skin-excision-stress.
n, t and P calculated according to the t-test of Student.
A.C.E. = Adrenal cortex extract. DOCA = Desoxycorticosterone acetate. Pre-lap. = Pre-laparotomy.

secretion, this phenomenon is not in complete accord with the concept of Ingle(4) that "the increased secretory activity of the adrenal cortices during stress tends to maintain homeostasis rather than to cause hypercorticalism."

With regard to the effect of adrenalectomy upon the healing process, previous reports have been contradictory(3,5). Our results indicate that adrenalectomy does not interfere with wound healing. This fact is itself of great interest. A number of authors have hypothesized that the negative nitrogen balance which follows wounding or surgical operation represents the mobilization of protein raw materials essential to the normal healing process(6-9). Adrenalectomy prevents this increased nitrogen excretion following stress(10-12). Nevertheless, in our experiment healing was not in the least impaired.

Summary. 1. The bursting pressures of healing laparotomy wounds, made 15 days after bilateral adrenalectomy were equal to or superior to those of normal control rats. 2. In adrenalectomized rats, maintained on a small fixed dosage of aqueous adrenal cortex extract, skin-excision-stress did not result in depression of the bursting pressure of 5th day laparotomy wounds, as is the case with non-adrenalectomized rats subjected to the same stress.

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Plasma Lipids in Fat Deficiency.* (21128)

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Essential fatty acid deficiency has been associated by different authors either with decreased lipid transport(1,2) or with increased lipid oxidation(3,4). In either case, the alteration should be reflected in the plasma lipids, a study of which should increase our knowledge of the fat deficiency disease. Surprisingly little information is available on the distribution of the plasma lipids in this disease. In most studies, the main subject of interest has been the changes occurring in the polyunsaturated fatty acids of the lipids. Some work has been done on the effect of low-fat diets fed for a limited time as distinct from fat-deficiency. Mellinkoff, Machella, and Reinhold(5) found that a low fat diet for 15 days was accompanied by a fall in serum cholesterol in 13 of 14 patients with lesions of the gastrointestinal tract. Recently, Wollaeger, Lundberg, Chipault, and Mason(6), in a study of 2 normal individuals, found that the total plasma fatty acids and cholesterol decreased somewhat during a period on a lipid-free diet. However, Brown, Hansen, McQuarrie, and Burr(7) found no change in the serum cholesterol or total fatty acids in one subject subsisting for 6 months on a diet extremely low in fat. Perhaps the only experiment in which the complete study of the effect of fat deficiency on the blood lipids was attempted is that of Hansen and Wiese(8). These authors studied at 3 months the serum lipids of dogs reared on a low fat diet from weaning. The results show slightly lowered total fatty acids, total cholesterol, and cholesterol ester (by difference) as compared with controls raised on the same diet plus lard.

Since a method recently developed in this laboratory makes possible the chromatographic separation of the plasma lipids(9), it was of interest to study their distribution in rats which had been on a fat-free diet from weaning and which showed definite signs of essential fatty acid deficiency. The results, which are described below, are compared with data secured similarly from fasted rats and from rats on a standard diet.

Experimental. Normal rats and rats which had been on a fat-free diet from weaning and had reached a growth plateau† were exsanguinated under ether anesthesia.‡ Extraction was carried out on plasma from individual animals or on pooled samples from several animals as described below. A total of 26 normal and 16 fat-deficient male Wistar rats was used in the experiment. Eighteen of the rats on the normal diet (Rockland Ray diet) were fasted 12-20 hours before being sacrificed. The conditions of extraction of the plasma lipids and the details of the chromatographic separations were as previously described(9) with 2 modifications.§

* This paper is based on work performed under contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

† Diet and conditions of establishing the state of fat deficiency described previously(10). Rats in this experiment had been on fat-free diet for 22 weeks when sacrificed. Average weight 236 g as compared with 370 g for controls on a Rockland diet.

‡ Note that upon centrifugation of blood of fat-deficient animals, the red cells hemolyzed to such an extent that the plasma, which in the normal animals was clear with a light yellow tinge, was deep red and almost opaque.

§ Fifteen column volumes of 1% ether in petroleum ether sufficed to elute the sterol esters. The mixture used to elute the triglycerides was altered to 3% ether in petroleum ether for following reason: Fillerup and Mead(9) were in error in stating that fatty acids followed cholesterol into the eluate. Fatty acids freed of the products of autoxidation and other impurities have been found to follow directly after the triglyceride fraction. Adequate separation was not possible, when 4% ether in petroleum ether was used as the eluant. However, by using 3% ether mixture in a few experiments, separations of palmitic and oleic acids from triolein were accomplished with recoveries of each component amounting

TABLE I. Plasma Lipid Composition of Normal, Fat-Deficient and Fasted Rats.

No. of rats and condition	Total lipid, mg %	Composition of lipid (upper figure—mg % range; lower figure—mean % of total ± stand. dev.)			
		Sterol ester	Triglyceride	Free sterol	Phospholipid
Normal	355	60–95	57–157	46–74	61–157
2, 2, 2, 1, 1	280–512	22.3 ± 3.5	31.9 ± 3.8	15.5 ± 2.7	24.3 ± 3.8
Fat-deficient	227	49–75	34– 57	14–40	62–101
5, 5, 6	176–275	29.0 ± 1.4	20.9 ± 5.8	9.9 ± 2.5	36.9 ± .9
Fasted	330	65–94	15– 43	21–51	123–199
6, 6, 6	245–463	26.2 ± 4.2	13.1 ± 1.7	11.4 ± 1.8	46.3 ± 2.1

Results. The composition of the plasma lipids of normal fed and fasted rats is compared with that of fat-deficient rats in Table I. It is apparent that despite considerable variability in the values, the total plasma lipid of fat-deficient rats is lower than that of normal fed or fasted animals. This result is in essential agreement with those of Hansen and Wiese(8).

However, when the values for the individual lipids are compared, it can be seen that this decrease is a result of decreases in the sterol ester, sterol and triglyceride fractions, whereas the phospholipid fraction remains normal. When the values for the fat-deficient animals are tabulated as percentages of the total plasma lipid, it is of interest to note that with one exception they are not significantly different (at the 0.95 level) from similar values calculated for normal fed or fasted animals. In other words, in fat deficiency, the several classes of plasma lipids have been lowered to about the same extent with the exception of the phospholipid fraction, which seems to be relatively increased. It is possible that these data demonstrate that in the fat deficiency state, the rate of fat metabolism remains constant, since it has been shown by Entenman *et al.*(11) that the plasma phospholipids are formed and utilized in the liver and that conversion to phospholipid is probably preliminary to oxidation of fatty acids in this organ. This may seem a surprising conclusion in view of the fact that these animals are on a fat-free diet unless it be recalled that animals in such a condition may actually deposit increased amounts of fat(12). We may also compare these results with those from the fasted animals, which are metabolizing their depot fats and which show an increased percentage of phospholipid, in agreement with previously published results(13-15).

to about 90%. No free fatty acid has been found in plasma lipids which have been treated with due care during extraction and subsequent handling.

Summary. The composition of the plasma lipids of fat deficient rats had been compared with that of normal fed and fasted animals using a recently developed chromatographic method. Total plasma lipid was about 64% of normal but the percent distribution of the various classes at this lower level was not significantly different with the exception of phospholipid which was relatively higher.

We wish to express our appreciation to Mr. W. H. Slaton of this laboratory for his assistance with the animals in this work.

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Physiological Action of 19-Norprogesterone in the Guinea Pig. (21129)

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Progesterone is the most potent antifibromatogen(1,2). However, there is no concomitant increase of the antifibromatogenic potency of testosterone when its progestational activity is increased by substitutions at C_{17} as with ethinyl-testosterone(3,4). Likewise, this progestational compound has no concomitant faculty to antagonize the hysterotrophic(3) or luteinizing action of estrogen (5).

It was of especial interest to examine whether the 3 mentioned actions (antifibromatogenic, antihysterotrophic, luteinizing) will increase with the progestational potency of derivatives of progesterone such as Δ^{11}-dehydroprogesterone(6) and 19-norprogesterone(7). The first of these compounds is 3 times, the second one 4 to 8 times as progestational as progesterone(6,8).

In a group of 30 animals we have found that Δ^{11}-dehydroprogesterone (Δ^{11}-dehydro-P) shares with progesterone (P) its antifibromatogenic and antihysterotrophic activity(9). But it is not certain whether antifibromatogenic potency of Δ^{11}-dehydro-P is greater than that of P; individual variations of the fibrous tumoral reaction in similar experiments and the error in calculation of quantities absorbed from subcutaneously implanted pellets especially when working with small quantities are considerable(10,11). The antihysterotrophic action of Δ^{11}-dehydro-P was certainly not greater than that of P. As we shall see the same result was obtained in new experiments. So far, we have not made a comparative study of the antiluteinizing potency of this compound.

Experiments. A group of castrated female guinea pigs were implanted subcutaneously with pellets of estradiol and Δ^{11}-dehydro-P, and another group with pellets of estradiol and 19-nor-P. In some experiments the pellets contained 40% of the specific steroid mixed with cholesterol, in others only 20%. This procedure, formerly used in experiments with P, assures absorption of very small amounts of the specific steroid. Classification of the fibrotumoral reaction was done as described previously(12).

Comparative results with P, Δ^{11}-dehydro-P, and 19-nor-P are given in Table I. This table shows that with Δ^{11}-dehydro-P the estrogen-induced fibrous effect is counteracted as with P. But, as found previously, with Δ^{11}-dehydro-P the antifibromatogenic activity was not strikingly superior to that of P (compare IV to II, and V to III); the difference was probably in the limit of the error (variation of the fibrous tumoral reaction; calculation of quantities absorbed). As to the antihysterotrophic activity Δ^{11}-dehydro-P was not superior to P.

With 19-nor-P both the antifibromatogenic and antihysterotrophic activity were considerably greater than those of P (compare VII to III).

The antiluteinizing activity of P can be expressed in the quantity necessary to counteract the luteinizing action of estrogen in intrasplenic grafts(5). With 16 to 23 μg per day absorbed from a 40% progesterone-cholesterol pellet frequency of corpora lutea is reduced

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TABLE I. Comparative Antifibromatogenic and Antihysterotrophic Activity of 3 Progestational Compounds in Guinea Pigs with Subcutaneously Implanted Pellets of Estradiol. Duration of experiments, 3 mo.

	Progestational steroid	No. of animals	Estradiol /day, μg	Progest. ster./day, μg	Fibrous tumoral effect†	Tumoral coeff. Q_{2-3}†	Uter. wt, g	Source of specific steroid No. of pellets, and percentage
I	Progesterone*	12	43	32 (21–47)	1.4 (.5–1.5)	0	2.1 (.9–2.7	
II	" *	16	37	15 (13–19)	1.4 (1 –1.5)	0	2.8 (1.7–5.1)	½–1, 40%
III	" *	12	40	9 (5–12)	3.6 (1.5–8)	1.0	3.2 (1.9–5.2)	
IV	Δ^{11}-dehydroprog.	4	44	14 (13–16)	2.0 (1.5–3)	.25	3.9 (3.1–4.5)	2, 40%
V	"	16	54	8 (5–11)	2.6 (1.5–5.5)	.44	3.2 (1.9–5.1)	½–2, 40%
VI	"	4	51	4 (3– 4)	2.6 (1.5–4)	.5	3.9 (2.7–6.4)	½, 40%
VII	19-norprog.	23	44	7.4(5–12)	1.6 (1 –3)	.1	1.5 (1 – 3.3)	1–2, 20%; ½, 40%
VIII	"	8	56	3.4(3– 4)	1.5 (1 –3)	.1	1.7 (1 – 2.4)	½, 20%

* Former experiments; for comparison only. See (9) and (13); with small corrections.
† Tumors of class 2 and 3. See for explanation (12).

from 90 to as little as 30%. In the present work with 12 to 23 μg of 19-nor-P per day absorbed from a similar pellet there was in 10 animals none with corpora lutea.

Discussion. Our comparative work with the 3 progestational compounds leaves no doubt that 19-nor-P is superior to P as to antifibromatogenic, antihysterotrophic, and antiluteinizing activity. Since as previously known, 19-nor-P is 4 to 8 times as progestational as P(8), 19-nor-P is superior to the latter is no less than 4 different functional aspects.

P also protects against toxic actions of estrogen, such as uterine bleeding and necrosis, which have been observed in guinea pigs receiving estrogen for several months(1;2, p. 118). Both Δ^{11}-dehydro-P and 19-nor-P have probably even greater capacity in this respect than P. In Group II with progesterone there were 2 cases with uterine necrosis; there was no uterine necrosis with Δ^{11}-dehydro-P or 19-nor-P.

On the basis of actual experimental knowledge one may reasonably ask why our body is using P and not 19-nor-P in matters of steroid homeostasis. One may venture that biosynthesis of 19-nor-P in ovarian structures would have been as easy as that of P.

As previously reported, calculation of absorption is subject to considerable error (10,11), particularly as calculation in the present work was based on the assumption that with 19-nor-P there was non-selective absorption from mixed pellets, as with P and those steroids for which non-selective absorption has been established(13-15). But our conclusion as to the high antifibromatogenic and antihysterotrophic activity of 19-nor-P remains unshaken even should we admit for this compound exceptionally selective absorption. Thus, in Group VIII the average weight of the pellet containing but 20% of 19-nor-P was 12.2 mg (range: 8.3-17.6). With selective absorption a maximum of 2.4 mg of 19-nor-P could have been absorbed in the course of 90 days, or 27 μg per day; an antifibromatogenic and antihysterotrophic action was obtained which was not inferior to that obtained with 21 to 47 μg of P in Group I.

Several years ago progesterone was unsuc-

cessfully used against uterine fibromyoma in women(16), possibly due to insufficient quantities of progesterone absorbed(2; p. 167). We feel that it would be of interest to repeat these clinical trials with 19-nor-P instead of P. Absorption from 19-nor-P pellets (pure, not mixed with cholesterol) is probably more rapid than from P pellets.

Summary. Antiestrogenic activities—antifibromatogenic, antihysterotrophic, antiluteinizing—of 19-norprogesterone have been compared to those of progesterone. 19-Norprogesterone, whose progestational activity is considerably greater than that of progesterone, is also strikingly superior as to antifibromatogenic, antihysterotrophic, and antiluteinizing action. On the contrary, the greater progestational potency of Δ^{11}-dehydroprogesterone compared to that of progesterone is seemingly not concomitant with greater antifibromatogenic and antihysterotrophic actions. The theoretical and practical bearings of the statements with 19-norprogesterone are discussed.

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Rauwolfia serpentina in Hypertensive Vascular Disease.*† (21130)

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In recent years, alkaloids of *Rauwolfia serpentina* have been found capable of producing hypotensive and sedative effects and bradycardia in animals and man. *Rauwolfia serpentina* (*Ophioxylon serpentinum*) is a tropical shrub growing throughout the Indian peninsula, the roots of which have been used for centuries for medicinal purposes. The hypotensive properties of the alkaloidal base extracted from this plant have been known since 1931(1), but clinical trials in hypertensive patients in this country were not carried out until a few years ago(2).

The present study was undertaken to throw further light on the clinical, circulatory and metabolic effects of *Rauwolfia serpentina* (Raudixin, Squibb) administered to patients

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TABLE I. Clinical and Laboratory Data of 4 Hypertensive Patients Treated with *Rauwolfia serpentina.*

Patient; Na and K intake, mEq/24 hr	Age, sex	Hosp. day	Dose, mg/d	Avg daily— Wt, kilos	"Resting" B.P., mm/Hg	Heart rate/min.	Avg urine output/24 hr Vol, ml	Na, mEq	K, mEq
J. DeZ.*	49 ♀	5	0	63.6	150/ 90	76			
Uncomplicated		10–15	0	64.0	140/ 88	77	1520	63	84
Na = 76		16–20	200	64.2	139/ 88	75	1540	60	85
K = 99		21–25	200	64.6	132/ 83	74	1470	59	85
		26–30	200	64.7	130/ 81	72	1540	65	89
		31–34	p†	64.8	133/ 83	74	1720	82	94
P. I.	54 ♂	8	0	71.2	180/110	78			
Cerebral arterio-sclerosis		13–18	0	71.1	162/106	82	1510	77	80
		19–23	200	70.8	167/105	80	1610	75	75
Na = 76		24–29	200	70.5	160/103	80	1120	60	82
K = 91		30–37	300	70.4	156/100	78	1190	84	93
		38–43	300	70.1	157/102	77	1180	62	71
		44–48	p	70.0	158/105	80	1160	72	74
		49–51	p	69.9	157/104	80	1060	67	75
S. R.	35 ♀	3	0	46.3	202/128	96			
Nephrosclerosis		8–13	0	46.8	212/122	85	1670	34	58
Na = 47		14–17	300	47.0	208/124	86	1570	20	54
K = 71		18–21	300	47.5	206/123	84	1530	23	65
		22–25	400	48.0	210/126	80	1960	41	59
		26–29	400	47.8	200/116	80	1930	52	52
		30–33	400	47.5	202/118	80	1880	59	67
		34–37	400	47.0	206/120	80	1530	38	65
		38–40	p	46.8	218/124	80	1610	48	65
		41–43	p	46.4	222/120	86	1330	42	63
D. U.	32 ♀	5	0	56.4	190/120	90			
Nephrosclerosis		10–15	p	56.1	214/128	88	1280	52	66
Na = 58		16–20	300	56.7	200/125	84	1250	30	51
K = 73		21–25	300	56.4	198/120	86	1390	64	64
		26–30	300	56.2	192/116	92	1440	66	67
		31–35	300	—	190/114	80	1120	69	52
		36–38	300	55.5	188/110	80	1510	66	66

* Casual B.P. prior to admission always above 200/120.
† p = placebo.

in various phases of hypertensive vascular disease.

Case material and methods. Clinical observations were made on 10 ambulatory patients with uncomplicated hypertensive vascular disease, and 10 bed patients, on the medical wards of the Presbyterian Hospital, with the accelerated ("malignant") phase of the disorder who showed proteinuria, some degree of nitrogen retention, and retinopathy with papilledema. Four additional patients (one uncomplicated, one with cerebral arteriosclerosis but no cardiac or renal involvement, and 2 with nephrosclerosis but no cerebral or cardiac involvement) were studied in more detail on the metabolic ward. The conditions of study, diagnostic procedures and methods employed in these 4 patients were similar to those reported previously(3), including the utilization of daily "resting" blood pressure measurements, constant dietary and fluid intakes, and preliminary periods of hospital supervision of 2-3 weeks. Known causes of hypertension, such as primary renal or adrenal disease and coarctation of the aorta, were excluded in all instances.

Results. 1. Raudixin, 50-100 mg twice daily, was given orally for one month to the 10 uncomplicated ambulatory patients, casual blood pressures having been recorded at weekly intervals for at least one month prior to therapy. Reductions in arterial tension of more than 15 mm Hg systolic and 10 diastolic (maximum 40 and 28) and in pulse rate of more than 10/min. were achieved in 7 instances within a 2-week period and maintained

throughout the period of drug administration, no significant changes occurring in the remainder. The only side-effect noted was slight nasal congestion in 4 patients. Four of those in whom a response was observed reported that they felt much less tense and nervous. The greatest effects were clearly in those individuals of the tense, hyperkinetic and emotionally labile variety. In no instance did the blood pressure fall to normal and in one patient several retinal hemorrhages appeared for the first time 6 weeks after the start of sustained treatment.

2. In contrast, Raudixin, 100 mg twice daily, administered for periods of 2-4 weeks to the bed patients in the accelerated phase, was without any subjective or objective effects. The casual blood pressure, measured twice daily, averaged 204/126 at the start of therapy and 207/128 at the close. The maximum systolic reduction was 12 mm, and diastolic 8. No significant changes in pulse rate were noted, and no patient showed any improvement in the degree of proteinuria or retinopathy or any decrease in the urea nitrogen concentration in the blood.

3. The results of the more detailed study undertaken in 4 patients are summarized in Table I. This group received Raudixin 100-200 mg twice daily for periods of 15-25 days. Two reported diminished nervousness and emotional tension, and one developed nasal congestion. Both patients with nephrosclerosis showed slight increases in weight during the early part of therapy followed by a decrease. "Resting" blood pressure values, compared to control readings, decreased in all patients to a minor extent, but never more than 10 mm systolic or 10 diastolic below the lowest values recorded in the control period. Changes in heart rate were unimportant and the urine volume fluctuations showed no consistent trends. The 2 subjects with nephrosclerosis exhibited sodium retention for periods of different lengths immediately following Raudixin administration (Fig. 1), subsequent urine sodium values being for a time in excess of the dietary sodium (Table I). The other 2 patients, both receiving a higher sodium content in their diets, reduced their urine sodium content to 39 and 50 mEq on the

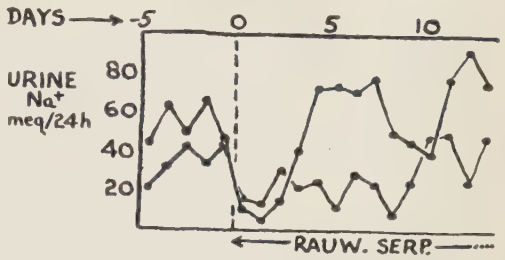

FIG. 1. Effect of Rauwolfia on sodium output in 2 patients with nephrosclerosis.

second day after starting the drug although the mean outputs for 5-day periods were not affected materially. No major alterations in potassium excretion were encountered.

No significant changes were noted in serum sodium, potassium, chloride, carbon dioxide content, urea nitrogen, fasting blood sugar, cholesterol and cholesterol esters, calcium and phosphorus concentrations. The hematocrit, hemogram and urinalysis remained unaltered and the cephalin flocculation test negative. Serial measurements of heart size by X-ray were unmodified, and weekly electrocardiograms and electroencephalograms were unaffected. The plasma volume, measured with the dilution technic employing I^{131}-labelled human serum albumin, increased 300 ml in one subject and decreased 100 ml in another. Under resting conditions, using the direct Fick principle with catheter in right atrium, the cardiac output remained 3.4 l/min. in one patient, rose insignificantly from 3.4 to 3.6 in the second. The splanchnic blood volume was determined in one instance with the change, from 894 to 963 ml, being within the limits of error of the method(4).

Discussion. The present study suggests that Raudixin may modify the casual blood pressure of some ambulatory patients with uncomplicated hypertensive vascular disease. It produces little change in those with the accelerated phase of the disorder or in hospital patients in varying stages of the hypertensive process after an adequate period of bed rest. At least in these short-term observations, rest or the superimposition of the accelerated phase appears to minimize any depressor effect. This observation, together with the absence of any apparent direct cardiac or circulatory action of the drug, lends support to

the concept that only a neurogenic component is modified(5). Whether the depressor action of Raudixin would be of greater benefit if the drug were continued for longer periods, or whether it will modify the natural history of the disease, cannot be stated from the present data. The appearance of retinopathy in one patient while under treatment is noteworthy.

The early and temporary urinary sodium retention, observed in 2 hospital patients with nephrosclerosis, was greater than could be explained by chance fluctuation in subjects on a constant fluid and electrolyte regimen; the transitory decrease in the 2 others may be of no significance. The relationship of *Rauwolfia serpentina* to sodium metabolism or the possibility that it may exert temporary effects on renal function will require further elucidation.

Raudixin seems to be a relatively safe drug and may be of value in the treatment of ambulatory hypertensive patients, particularly those who are tense, hyperkinetic and without complications. Its influence on the course of hypertensive vascular disease remains to be established.

Summary. 1. *Rauwolfia serpentina* (Raudixin Squibb) was administered for periods of 2 weeks or more to 10 ambulatory patients with uncomplicated hypertensive vascular disease, 10 bed patients with the accelerated phase of this disorder, and to 4 hospital patients in varying stages of the disease, the last group being studied in detail while on a constant fluid and dietary regimen. 2. Significant depressor effects and bradycardia were observed in the majority of the ambulatory patients; some degree of relaxation was noted in those who were emotionally tense; but little or no effects were evident in those with the accelerated phase or those under hospital supervision. 3. Temporary sodium retention was apparent in 2 hospital patients with nephrosclerosis, but otherwise Raudixin appeared to have no direct hemodynamic, circulatory or metabolic action.

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Phagocytosis Influenced by Growth Media, Filter Paper and *para*-Hydroxybenzoic Acid. (21131)

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Earlier studies(1) demonstrated greater phagocytosis of *Escherichia coli* grown in beef infusion medium than when grown in tryptose medium. Phagocytosis of killed *E. coli* was increased also by previous treatment with filter paper or *para*-hydroxybenzoic acid(2). The latter compound, an active principle in filter paper, was reported by Davis(3,4) as a new bacterial vitamin and antagonist for *para*-aminobenzoic acid. These observations have been extended to *Brucella abortus.*

Materials and methods. Culture media. *Brucella abortus* strain 19 was kept routinely on Bacto Tryptose agar enriched as noted below, and inoculations were made from 24-hour cultures into the media to be tested. In the initial studies on phagocytosis of *Brucella* grown in beef and tryptose broth, the stock inoculum was carried in tryptose broth and transferred every 24 hours. When daily transfers of the stock culture were made in this broth medium, phagocytosis of both beef and tryptose grown bacteria by neutrophiles from normal guinea pigs under standardized

conditions gradually increased from the levels of about 55% usually seen to 94% levels in 2 weeks. Exposure of these animals to *Brucella* was excluded as a cause when blood samples from another group of guinea pigs raised and maintained in a laboratory free from possible contact with *Brucella* showed similarly elevated phagocytic activity. Phagocytosis of a standardized killed suspension of *Brucella*(5) was at normally low levels in the blood of the same animals; thus it appeared that a change in the microorganism was responsible for the above result. Plating the broth cultures of *Brucella* revealed 20% of rough mutant colonies, which were apparently more susceptible to phagocytosis. This change in the microorganism was prevented in subsequent experiments by inoculating the media to be tested from 24-hour stock cultures grown on tryptose agar. A comparison of phagocytosis of *B. abortus* was made between microorganisms grown in Bacto Tryptose broth or agar enriched with 10 g dextrose, 10 mg $FeSO_4$, and 0.1 mg thiamine hydrochloride per l, and those grown in Bacto Beef infusion broth or agar containing 20 g peptone and 5 g NaCl per l. To augment growth, the broth media were used in shallow layers of 25 ml in 250 ml Erlenmeyer flasks. Similar growth augmentation could be obtained in wide tubes agitated during incubation. The bacteria were grown for 20-24 hours at 37°C, harvested by centrifugation or by taking up in saline, washed in 0.9% saline, and resuspended in saline to a concentration of 1 x 10^{10} per ml. The density of the suspensions was measured turbidimetrically with a Coleman Model 11 spectrophotometer, and checked by the dilution plate count method, using the "drop plate" technic of Reed and Reed(6), and Pomales-Lebron and Fernandez(7). For the experiments with filter paper and *para*-hydroxybenzoic acid, the bacteria were grown for 24 hours at 37°C on tryptose agar enriched as noted above, and concentrated to 1 x 10^{10} per ml. The bacteria were killed by treatment with 1% CH_2O for 24 hours at room temperature, washed 3 times in saline, brought to the same concentration, and sterility was checked by plating.

Filter paper experiments. Strips measuring 1 x 4 or 1 x 5 cm of Whatman No. 1 or No. 42 filter paper were rolled and placed in the bottom of 12 ml tapered centrifuge tubes. Dead bacteria contained in 0.5 to 0.8 ml of suspension were pipetted into these tubes and allowed to remain in contact for one to 2 hours, after which the filter paper was squeezed out with glass rods or pipettes and removed. In previous experiments(2), chamber counts showed no significant loss of bacteria after this treatment. *Para-hydroxybenzoic acid experiments.* Killed bacterial suspensions were centrifuged, the saline removed, and the sedimented dead bacteria were suspended in aqueous solutions of Eastman C.P. *para*-hydroxybenzoic acid containing from 0.01 to 1.0 mg per ml. The volumes of suspending solutions were 1, 2, and 6 ml in different experiments, but the concentration of microorganisms exposed to *para*-hydroxybenzoic acid was maintained at 3 x 10^9 per ml. After 1-2 hours in contact, the bacteria were centrifuged, and resuspended in saline to a concentration of 1 x 10^{10} per ml. Controls for the filter paper and *para*-hydroxybenzoic acid-treated experiments were saline-washed microorganisms.

Phagocytosis. The bacterial suspensions contained 1 x 10^9 live or killed *Brucellae* in 0.1 ml of saline. In the growth medium experiments, heparinized guinea pig blood was used. In the experiments with filter paper and *para*-hydroxybenzoic acid, dog blood cells were used after 6 washes in Krebs-gelatin solution(5) and suspension in an equal volume of Krebs-gelatin. Equal volumes of blood cell and *Brucella* suspensions were mixed and rotated for 30 minutes at 37°C. Smears of these rotated mixtures were prepared, stained with Giemsa-Jenner, and the percentage of 50 neutrophiles containing *Brucella* was tallied.

Results. Effect of growth media. In 19 observations, the mean percentage and standard error of neutrophiles phagocytosing live *B. abortus* was 58.7 $\pm$ 4.8 when the organism was grown in tryptose broth, and 53.1 $\pm$ 4.1 when the organism was grown in beef infusion broth. The mean percentage difference and its standard error, 5.6 $\pm$ 6.30, was not statistically significant. In 26 observations, the mean percentage and standard error of neu-

trophiles phagocytosing live *B. abortus* was 52.0 ± 4.8 when the organism was grown on beef infusion agar, and 67.8 ± 3.6 when the organism was grown on tryptose agar. The mean percentage difference and its standard error, 15.8 ± 5.98, was statistically significant. The susceptibility of the bacteria to phagocytosis was the same when grown in tryptose broth, beef infusion broth, or beef infusion agar, but was increased when they were grown on tryptose agar.

Effect of filter paper contact. In 66 observations, previous contact with filter paper resulted in a mean percentage and standard error of 33.3 ± 1.1 of neutrophiles phagocytosing killed *B. abortus,* compared to 22.6 ± 1.2 for saline-treated controls. The mean percentage difference and its standard error, 10.7 ± 1.57, was statistically significant. Whatman No. 1 and No. 42 papers, compared in 33 experiments, had equivalent stimulatory effects.

Effect of contact with para-hydroxybenzoic acid. Previous contact with *para*-hydroxybenzoic acid in concentrations of 0.01, 0.1, and 1.0 mg per ml, in 99 observations showed a mean percentage and standard error of 34.3 ± 0.9 of neutrophiles phagocytosing killed *B. abortus,* compared to 22.6 ± 1.2 for the saline-treated controls. The mean percentage difference and its standard error, 11.7 ± 1.47, was statistically significant.

The stimulatory effect of *para*-hydroxybenzoic acid was greater with increasing concentration. In 33 observations, the mean percentage and standard error of neutrophiles phagocytosing killed *B. abortus* after contact with 1.0 mg per ml of *para*-hydroxybenzoic acid was 37.9 ± 1.6, with 0.1 mg per ml was 34.2 ± 1.3, with 0.01 mg per ml was 30.9 ± 1.4, and after contact with saline was 22.6 ± 1.2. The per cent of neutrophiles phagocytosing bacteria could be plotted as a linear function of the logarithm of the concentration of *para*-hydroxybenzoic acid employed.

Discussion. *B. abortus* 19 was sensitized for phagocytosis by growth on tryptose agar medium, but not by growth on beef infusion agar, tryptose broth, or beef infusion broth. These results differed from those observed with *E. coli*(1), which were phagocytosed more readily when grown in beef broth or agar than when grown in tryptose broth or agar.

Stained smears and turbidity measurements of live suspensions indicated that *Brucellae* grown on tryptose agar were slightly smaller than those grown on beef infusion agar. On the basis of Fenn's observations that large particles were phagocytosed more readily than small ones(8), the greater degree of phagocytosis of *Brucella* grown on tryptose agar could not be attributed to size. This discrepancy between size and phagocytosis was also noted in the case of *E. coli*(1), where the smaller bacteria grown in beef media were more readily phagocytosed than the larger cells grown in tryptose media.

Thus, the *in vitro* phagocytosis of *Brucella abortus* as well as *E. coli* could be influenced by the medium in which it was cultivated, and the different effects of media with different microorganisms emphasize the importance of control of growth media in the study of these phenomena.

The stimulation of phagocytosis of killed *E. coli* suspensions by filter paper and by *para*-hydroxybenzoic acid reported previously (2) was observed also with *B. abortus* strain 19. This was a direct effect upon the bacteria, since these materials could not have affected the leukocytes under the conditions of the experiments.

Summary. 1. The medium in which *B. abortus* strain 19 was grown influenced the phagocytosis of this microorganism. *Brucellae* grown on tryptose agar were phagocytosed more readily than those grown on beef agar, or in tryptose or beef broth. The effects of the media were different with *Brucella* and *E. coli.* 2. Phagocytosis of formalin-killed *B. abortus* 19 was promoted by previous contact with filter paper or *para*-hydroxybenzoic acid. The latter was effective in concentrations of 1.0 to 0.01 mg per ml, and the stimulatory effect was related in linear fashion to the logarithm of the concentration of *para*-hydroxybenzoic acid. These effects of filter paper and *para*-hydroxybenzoic acid with killed microorganisms were similar to those observed with *E. coli.*

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Effect of Sodium Thiosulfate Upon Mustard-Virus Interaction.* (21132)

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It was previously reported that exposure of influenza virus to appropriate concentrations of mustard resulted in selective inactivation of certain viral attributes such as infectivity and toxicity(1). In analyzing the early stages of destruction of viral toxins by mustard, it was necessary to circumvent the inherent toxicity of mustard itself. The use of sodium thiosulfate in this connection resulted in a rapid detoxification of mustard by thiosulfate(2). This observation along with previous reports of mustard-thiosulfate interaction(3) suggested a possible influence of thiosulfate upon inactivation of virus by mustard. This paper reports the results of such studies.

Materials and methods. Virus. Influenza A (PR8) virus was prepared as described(2). Harvested virus was stored in ampoules in a dry-ice cabinet. When needed, an ampoule of virus was placed in a 37°C water bath for 30 minutes. Following this, the viral suspension was centrifuged 15 minutes at 3500 rpm; the supernatant fluid was removed, allowed to attain room temperature and then used as the source of virus. *Mustard.* The chloroethylamine derivative used in these investigations was cyclohexyl [bis(β-chloroethyl)] amine hydrochloride, kindly supplied by Dr. H. B. Woodruff of Merck and Co., Inc. The chemical solution was prepared in triple-distilled water to yield for the 0.01 M solution of mustard a final pH of 2.6. *Thiosulfate.* Freshly prepared solutions of sodium thiosulfate in distilled water were used.

Exposure of virus to mustard. This was carried out as previously described(2) except that the reaction temperature in these experiments was 25°C. In testing for the effect of thiosulfate upon these mustard-virus systems, sufficient 3 M sodium thiosulfate was added to the samples (before addition of mustard) to yield a final thiosulfate concentration of 0.075 M. *Test of viral toxicity.* Serial 2-fold dilutions of mustard-treated virus were made in normal allantoic fluid; these were tested for residual viral toxicity by injecting 1.0 ml of dilution intravenously into a mouse, 6 mice being used for each dilution. The animals employed in the tests were Swiss mice (Webster strain) 12 to 15 g weight and of random sex. All animals were observed daily for a period of 7 days, and deaths occurring 24 hours or later after intravenous injection were considered to be of viral origin. *Infectivity test.* This was carried out as described previously(2).

Results. Effect of thiosulfate upon destruction of viral toxicity and infectivity by mustard. The effect of sodium thiosulfate upon mustard-virus interaction is shown in Table I. The results presented in this table are representative of a series of similar experiments.

In terms of mustard inactivation of viral toxicity, it is apparent that there was less destruction of viral toxin in the presence of sodium thiosulfate. The greater residual toxicity of virus exposed to mustard in the pres-

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TABLE I. Effect of Sodium Thiosulfate in Mustard Inactivation of Virus.

Sample	(M) Thios.	(M) Mustard	Toxicity of sample* u	1:2	1:4	Neg log ID_{50} remaining
Virus	.075	5.0×10^{-4}	1/6			4.7
"	"	2.5 "	4/6			6.3
"	"	1.25 "	6/6	4/6	0/6	7.3
"	"	6.25×10^{-5}	6/6	4/6	0/6	7.3
"	0	5.0×10^{-4}	0/6			<1.0
"	"	2.5 "	0/6			4.7
"	"	1.25 "	1/6	0/6		5.7
"	"	6.25×10^{-5}	5/6	1/5	0/6	7.3
"	"	0	6/6	6/6	1/6	8.3
"	.075	0	5/5	6/6	1/6	8.5
NAF	0	5.0×10^{-4}	0/6			
"	.075	"	0/6			

NAF = Normal allantoic fluid.
(M) = Final molar concentration.
Blank spaces indicate samples not tested.

* Samples were left 3 hr at room temp. and 18-20 hr in refrigerator before tests for residual viral toxicity and infectivity. The 1:2 and 1:4 dilutions of samples were made in NAF. u represents undiluted sample.

ence of thiosulfate was most evident when 1.25 x 10^{-4} M mustard was used. These deaths are properly attributable to viral toxicity, for normal allantoic fluid treated with mustard alone or in the presence of thiosulfate proved innocuous upon intravenous injection of mice. The "sparing" action of 0.075 M sodium thiosulfate upon mustard inactivation of virus extended also to viral infectivity. Thus, for the 3 highest concentrations of mustard, destruction of viral infectivity, as measured by the log number of egg-infective doses remaining, was approximately 1.6 to over 3.7 log units greater in the absence of thiosulfate.

Effect of thiosulfate upon rate of destruction of viral infectivity by mustard. The conclusions derived from Table I represent the end results of mustard action.. An earlier report(2) had indicated the extreme rapidity of mustard-virus interaction. To determine the effect of thiosulfate upon the rate of reaction between mustard and virus, the experiments shown in Table II were carried out.

It is evident that thiosulfate had little influence upon the course of events occurring during the first minute of reaction. Addition of mustard to virus resulted in an approximate drop of 3 log units irrespective of the presence or absence of thiosulfate. The effect of thiosulfate upon this system became apparent only after the first minute of reaction; thus, while a further drop in viral infectivity (approximately 2 log units) occurred in the next 4 minutes in the mixture without thiosulfate, no additional loss was detected in the system containing 0.075 M thiosulfate. In other experiments (results not shown) using 5 x 10^{-4} M mustard, it was found that an increase in the concentration of thiosulfate from 0.075 M to 0.5 M still failed to prevent a marked initial drop (*i.e.* in the first minute) in viral infectivity upon addition of mustard to virus.

Discussion and summary. The present studies would appear to indicate that the rate of reaction of mustard with virus was extremely rapid and that there were chemical groups in influenza virus which rendered them highly susceptible to mustard action. The rapidity of reaction at room temperature was attested by the observation that the greatest portion of the viral population was inactivated during the first minute of mustard-virus interaction. The inability of sodium thiosulfate, a substance reputedly having marked affinity for mustard molecules, to influence the course of events during the first minute of reaction seemingly supported the second of the above premises; presumably thiosulfate failed to compete successfully with virus for the molecules of mustard, thus accounting for the rapid initial destruction of virus. The partially "protective" action of thiosulfate upon destruction of virus by mustard, as reported herein, was confined to events after the first minute of reaction, and was probably related to a tardy combination of thiosulfate and mustard molecules.

TABLE II. Effect of Thiosulfate upon Initial Stages of Mustard-Virus Interaction.

Viral sample	(M) Mustard	(M) Thios.	Time of sampling (min.)	Log ID_{50} of sample
1	5×10^{-4}	.075	1	5.7
2	"	"	5	5.5
3	"	0	1	5.5
4	"	"	5	3.7
5	0	"		8.7
6	"	.075		8.5

Reaction was at room temp.

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Effect of Hyaluronic Acid on Skin Lesions Produced by Staphylococci.* (21133)

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The increasing interest aroused by recent biochemical and enzymological researches on the mucopolysaccharides of the connective tissue ground substance has led us to carry out a series of investigations on the significance of such substances in inflammation processes and experimental carcinogenesis. In this first paper some observations are reported on the action of hyaluronic acid and its enzymatic split products on an inflammation experimentally produced in the rabbit skin by inoculation with pathogenic staphylococci. These investigations follow the observations of Scott *et al.*(1) who reported a slight shortening of incubation period in experimental syphilis, if spirochetes are inoculated in the rabbit testis together with hyaluronidase. According to these authors, this is due either to direct action of hyaluronidase on spirochetes or on the ground substance. On the other hand, evidence has been repeatedly given (Meyer and Chaffee(2), Campani(3), Caputo(4), Cutinelli(5), McClean *et al.*(6)) of the participation of mucopolysaccharides of the connective tissue ground substance in inflammation processes and in the formation of granulation tissue (Caselli(7), Baggi (8-12)).

Methods. The following materials have been used: A) 24-hr agar cultures of *Staphylococcus aureus.*† Immediately before the experiment the bacterial growth of each culture was suspended in 10 ml of saline. B) Potassium hyaluronate solution (7.5 mg/ml) in saline, solution pH 7.3.‡ C) Split products of potassium hyaluronate obtained by incubating for 2 hours at 37°C the solution listed as B with hyaluronidase§ (10 Schering units/1 ml of hyaluronidase solution), then inactivating the enzyme by keeping mixture in water bath at 80°C, 30 minutes. D) 24-hr agar cultures of the same strain of *S. aureus* A, seeding from a broth culture after 5 successive cultures in broth (4 ml) to which one ml potassium hyaluronate had been added at concentration of 2 mg/ml. The fifth broth culture was then seeded in agar and the growth suspended in 10 ml saline. Experiments were made on 9 albino rabbits from the same breed, weighing approximately 2.5 kg. The back was carefully shaved, not to produce abrasions. Each animal was given 2 intradermal injections on both sides at 8 cm distance from each other. The animals were divided into 3 groups: *1st group.* 4 rabbits injected in left side skin with 0.4 ml (2 animals) and 0.2 ml (2 animals) of a mixture made with equal volumes of bacterial suspension A and potassium hyaluronate solution B. Controls were injected in the right side with the same amount of a mixture containing the bacterial suspension and saline in equal volumes. *2nd group.* 3 rabbits were injected in the left

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† A strain of staphylococcus has been used recently isolated from an abscess.

‡ We wish to express our gratitude to Dr. H. Gibian of the Schering Laboratory of Berlin for his kind supply of hyaluronate.

§ Throughout our experiments hyaluronidase (Kinaden) has been used prepared by the Schering Laboratory, Berlin.

TABLE I. Inflammation Areas in mm² Produced by Intradermal Inoculation of a Mixture of Staphylococci and K Hyaluronate, and by a Control Mixture of Staphylococci and Saline.

*	1†			5†			9†			11†		
ml	A	B	△	A	B	△	A	B	△	A	B	△
0.4	567	273	294	223	50	173	196	0	196	190	0	190
	693	532	161	400	81	319	111	0	111	111	0	111
0.4	858	180	678	1189	101	1088	310	40	270	250	40	210
	1008	396	612	989	50	939	756	44	712	528	40	488
Mean	781	338	436	700	70	629	343	21	322	269	20	249
0.2	255	96	159	129	27	102	89	0	89			
	610	48	562	200	16	184	69	0	69			
0.2	49	34	15	133	29	104	51	22	29	31	7	24
	139	31	108	286	30	256	71	29	42	81	11	70
Mean	263	52	211	187	25	161	70	12	57			

* Amount of injurious agent used.

† Days elapsed between inoculation of injurious agent and evaluation of inflammation area in mm².

A—Inflammation area produced by inoculation of staphylococci and K hyaluronate.

B— " " " " " staphylococci and saline.

△—Difference in mm² between the 2 inflammation areas.

side with 0.2 ml of mixture containing equal volumes of bacterial suspension and the solution of potassium hyaluronate split products C. Controls were injected in the right side with the same amount of a mixture containing bacterial suspension and saline in equal volumes. *3rd group.* 2 rabbits were injected in the left side with 0.2 ml of staphylococci suspension grown in potassium hyaluronate, D. Controls were injected in right side with the same amount of a staphylococci suspension previously cultivated in plain broth.

In order to make sure that no independent inflammatory action was induced by either potassium hyaluronate or its split products, these materials were injected in quantities from 0.1 ml to 0.5 ml into the skin of 2 more rabbits. The extension of the inflammation areas was daily kept by drawing their outline on cellophane and calculating the result in square millimeters. The results are shown in the Tables and Figures. The skin lesion produced in animals of first, second and third groups was represented by a well-defined papular zone. On the third day following injection of the injurious agent, rabbit No. 2 showed the formation of large crusts on the center of the papular lesions.

The results obtained from the first group (see Table I and Fig. 1) give a clear demonstration that addition of potassium hyaluronate to the staphylococci brought on a constant remarkable intensification of the inflammatory reaction. This is particularly evident in animal 2 of Table I (see Fig. 2). For on the 5th day following injection of the larger dose of the injurious agent, the inflammation

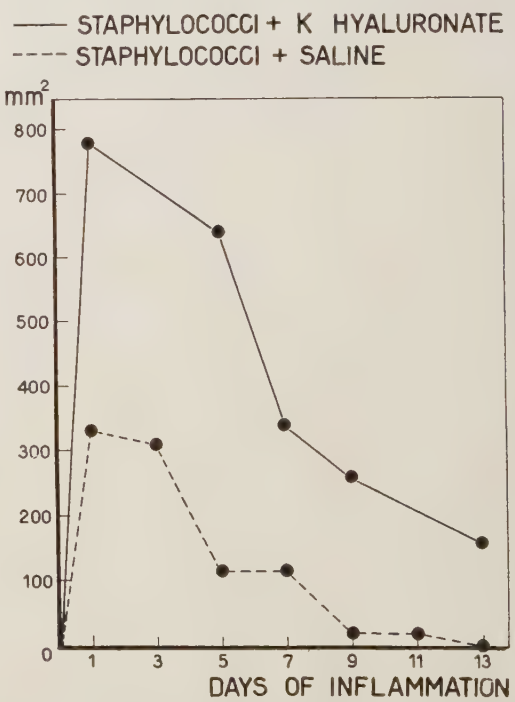

FIG. 1. Average measure of the inflammation areas in rabbits. 1-2 of first group.

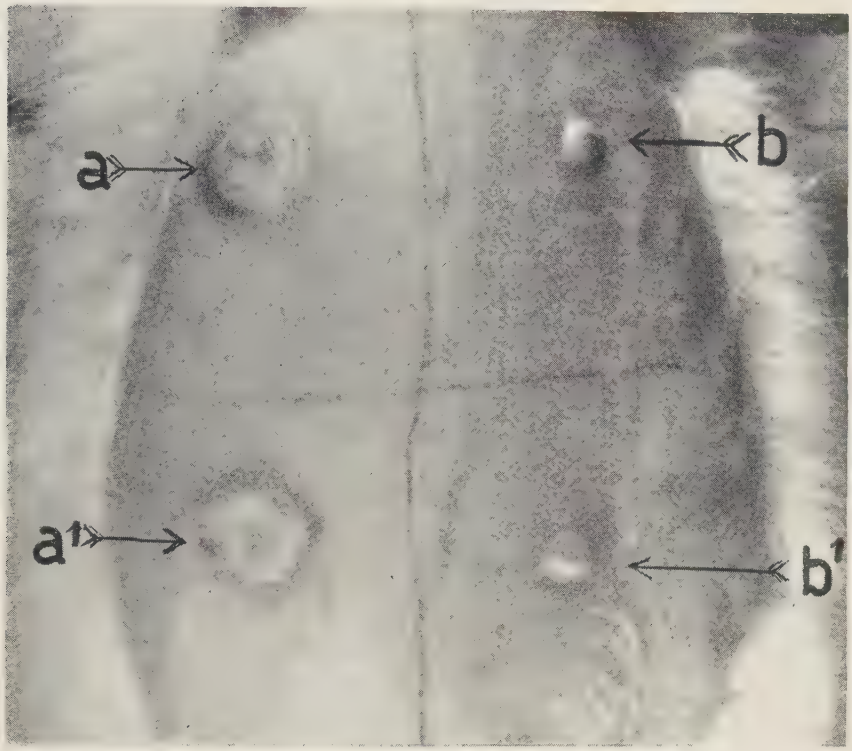

FIG. 2. Lesions produced by staphylococci + K hyaluronate, and control lesions produced by staphylococci + saline. A, 0.4 ml staphylococci + K hyaluronate; B, 0.4 ml staphylococci + saline; A^1, 0.2 ml staphylococci + K hyaluronate; B^1, 0.2 ml staphylococci + saline.

area measured 1189 mm^2 in comparison to 101 mm^2 of the control area, while the lesion induced by the smaller dose of staphylococci had an area of 989 mm^2, the control only 50 mm^2.

The addition of hyaluronic acid split products to the staphylococci likewise produces an increased cutaneous inflammatory reaction, though in much slighter degree than by addition of hyaluronate. The greatest differences between the inflammation areas are represented by rabbit 1 on the third day after injection of causative agent and by rabbit 2 on the fifth day: the former showing 117 mm^2

TABLE II. Inflammation Areas in mm^2 Produced by Intradermal Inoculation of a Mixture of Staphylococci and K Hyaluronate Split Products and by a Control Mixture of Staphylococci and Saline.

*	1†			3†			5†			7†			9†			11†			13†		
ml	A	B	△	A	B	△	A	B	△	A	B	△	A	B	△	A	B	△	A	B	△
0.2	76	72	4	117	85	32	47	20	27	58	32	26	37	13	24	18	6	12	15	0	15
	78	65	13	128	58	70	79	23	56	50	22	28	43	11	32	30	13	17	10	0	10
0.2	247	120	127	86	72	14	109	36	73	88	39	49	49	24	25	35	14	21	22	0	22
	105	81	24	71	41	30	75	35	40	96	35	61	41	26	15	51	13	38	18	5	13
0.2	280	132	148	187	102	85	146	100	46	95	40	55	32	19	20	11	0	11			
	90	115	25	173	70	3	65	62	3	55	30	25	21	9	12	7	0	7			
Mean	146	97	48	110	88	39	86	46	40	73	39	40	37	17	21	25	7	17			

* Amount of injurious agent used.
† Days elapsed between inoculation of injurious agent and inflammation area in mm^2.
A—Inflammation area produced by inoculation of staphylococci and K hyaluronate split products.
B— " " " " " " staphylococci and saline.
△—Difference in mm^2 between the two inflammation areas.

vs. 85 mm^2 and 128 mm^2 vs. 58 mm^2, while the latter showed 109 mm^2 vs. 36 mm^2 and 75 mm^2 vs. 35 mm^2 (Table II).

The inflammatory lesions produced by the staphylococci cultivated in presence of potassium hyaluronate showed no significant differences in comparison with the control lesions produced by staphylococci cultivated in normal media. Neither potassium hyaluronate nor its split products induced any appreciable lesion in the 2 rabbits inoculated as controls.

No definite statement can be made about the mechanism with which hyaluronic acid, and in lesser degree its split products, caused the remarkable spreading of inflammation processes. We assume that the mucopolysaccharide, by causing alterations of the tissue where it was inoculated together with the staphylococci, possibly originated interactions between these microorganisms and the tissue, thus favoring the development of the lesions.

Summary. Intradermal inoculation of a mixture of K hyaluronate and staphylococci constantly produces larger lesions than by staphylococci alone. A similar but slighter increase in lesions is also observed after inoculation of a solution containing staphylococci and K hyaluronate split products.

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A New Method for Direct Recording of Cardiac Output.* (21134)

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No simple method is available to measure under physiological conditions directly and accurately total blood flow in the main pulmonary artery. It is the purpose of this communication to report a method for the determination of cardiac output which makes it possible to measure directly in experimental animals main pulmonary artery flow and its fluctuations from one heartbeat to the next. This has been accomplished with the aid of a bristle flowmeter(1-3) which is suitably modified for use in the pulmonary artery trunk.

Method. Fig. 1 shows a diagram of the flowmeter cannula. The device consists of 3 brass parts (A,B,C) which are assembled by threaded joints. The tip of part A is inserted into the vessel wall by slipping the lip D through a "button hole" opening in the artery wall using a technic similar to that employed with the non-suture aortic shunt developed in this laboratory(4,5). The lip D is secured firmly to the intima by the pressure of plate E on the outside of the wall. Plate E is held in place by the screw F. In the shaft of part A lies a snugly fitting stainless steel cylinder G, which can be pushed forward or retracted by means of a cable release H, suitably attached to the outside of the cylinder. When the cylinder G is in the retracted position as shown in Fig. 1, the bristle I, protruding into the lumen of the pulmonary artery, can be

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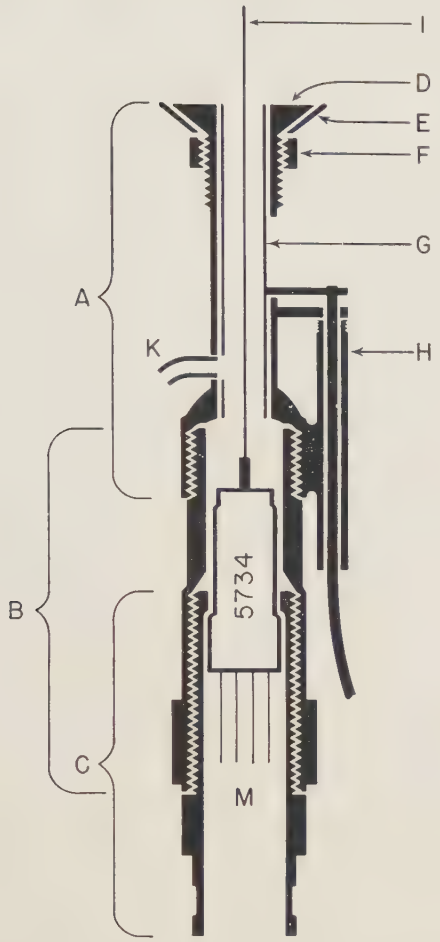

FIG. 1. Diagram of "bristle-flowmeter" modified for use in the pulmonary artery trunk for cardiac output determination. Total length of cannula, 75 mm. Description in text.

deviated by the blood stream. When the cylinder G is pushed forward it advances one mm beyond the tip of the bristle I and protects the bristle from deviation by the blood current. This device for producing "zero flow" makes it possible to check on electronic zero drift at frequent intervals. The small diameter of the cylinder (4 mm) precludes any significant obstruction to blood flow during the zeroing maneuver. Since the cable release H is 20 cm in length, the zeroing device can be operated from the outside of the animal after chest closure. A stainless steel tube K (13 gauge hypodermic needle) is attached at the side of part A for obtaining pulmonary artery pressures and for removal of air bubbles. Part B is a long-threaded shaft, into which part C can be advanced by turning. Part C serves as a socket for the RCA 5734 vacuum tube mechano-electrical transducer. Deviation of the bristle I results in an electrical signal which is transmitted by the electrical leads M to a recording instrument (1-3).

The placement of the flowmeter in the pulmonary artery trunk is illustrated in Fig. 2. The chest was entered at the fourth left intercostal space and the pericardium incised for a distance of 4 cm over the pulmonary artery trunk, leaving the heart in the pericardial sac. A string was passed around the main pulmonary artery. About one-quarter of the anterior pulmonary artery wall was gently clamped in a longitudinal direction with a modified Potts clamp, thus creating a dry field for a button hole incision of the wall. The clamping interfered only slightly with the maintenance of pulmonary flow judging from the aortic blood pressures. The bristle had

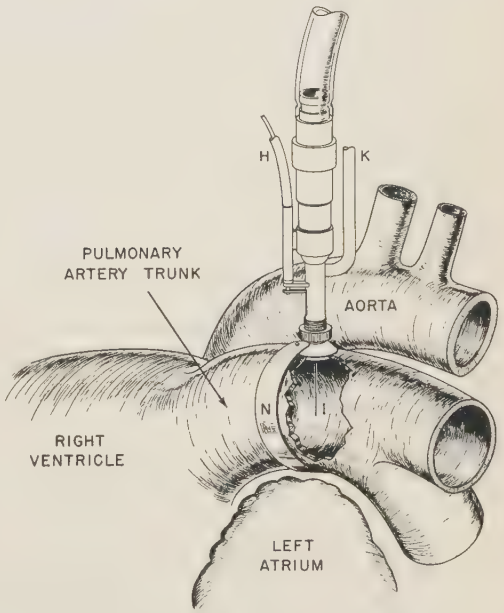

FIG. 2. Diagram of the "bristle-flowmeter" illustrating its position in the pulmonary artery. H = Cable release, K = Pressure recording tube, I = Bristle, N = Metal band, which fixes the circumference of the pulmonary artery trunk by passing around the vessel. The zeroing cylinder is not visible because it is retracted.

to be protected from damage during the insertion. This was accomplished by turning part C in the threads of part B until the tip of the bristle was retracted behind the lip D of part A (Fig. 1). Then lip D was slipped into a button hole incision in the clamped anterior wall of pulmonary artery without blood loss. Slipping of the lip from the vessel wall was prevented by closing the incision around the cannula tip with 5-0 silk suture, although this is not essential. Then plate E was pressed on the adventitia. After releasing the Potts clamp the tip of the bristle was advanced 1 cm into the lumen of the pulmonary artery trunk by turning part C in the threads of part B. A 4 mm wide stainless steel metal band N was drawn around the pulmonary trunk with the aid of the previously placed string and then securely fastened with 2 snaps to plate E (Fig. 2). This metal band stabilized the vessel diameter, which was indispensable for volume flow measurements. The length of the band was chosen according to the vessel's diameter; it fitted snugly to the outer wall during diastole without restricting flow. In several experiments the chest was closed after the flowmeter insertion and normal intrathoracic pressures were reestablished. The flowmeter was calibrated *in situ* with the animal's own blood after each experiment. For calibration, blood entered the pulmonary artery from a cannula, which was tied into the pulmonary conus, and was allowed to leave through another cannula ligated into the bifurcation of the right and left pulmonary arteries.

Ten experiments were performed in heparinized dogs weighing from 17 to 28 kg. The animals were anesthetized with 3 mg/kg of morphine sulfate subcutaneously and 30 mg/kg sodium pentobarbital intravenously. Simultaneously with pulmonary artery flow, optical records were taken of aortic, right atrial, and main pulmonary arterial pressures. The pressures were recorded with modified Gregg manometers using established methods of this laboratory(2).

Results. Fig. 3 shows a segment of an original record illustrating the phasic flow changes in the pulmonary artery during the heart cycle. Ejection from the right heart occurred almost simultaneously with the rise of aortic pressure and terminated at the time the incisura was reached. The flow rate at the peak of ejection was 5300 cc/min. Ejection was followed by a brief period of backflow marked X which reached a rate of 1500 cc/min. Backflow was a consistent finding in all experiments. The backflow is presumably caused by the backward movement of blood into the proximal portion of the pulmonary artery trunk when the semilunar valves bulged into the right ventricle during their closure. It is possible that some of the backflow reached the right ventricle during the process of valve closure. A slight forward movement of blood occurred during the early part of diastole. This is borne out in the record of Fig. 3 by the slight forward flow during early diastole, marked y. Flow then came essentially to a halt until the next ejection.

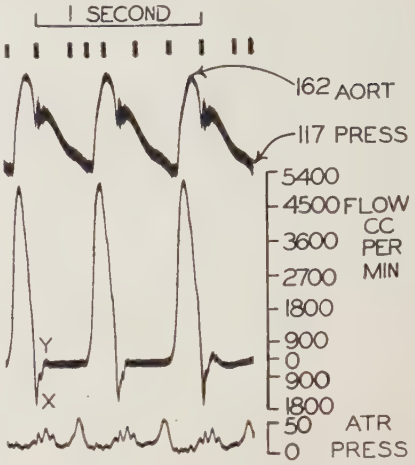

FIG. 3. Cardiac output phasically recorded with the bristle flowmeter illustrated in Fig. 1 and 2. (Dog, 18 kg weight, heart rate 120, cardiac output 862 cc.) Tracings from top to bottom: Time, aortic pressure in mm Hg. Flow in pulmonary artery trunk in cc/min., right atrial pressure in mm H_2O. (⅓ of original.)

The stroke volume can be calculated by measuring the area under the flow curve. Table I shows the values of forward and backward flow during each of the 3 cardiac cycles depicted in Fig. 3. Cardiac output calculated from these values amounted to 862 cc. To obtain correct values, backward flow must be deducted from forward flow. It is

TABLE I. Stroke Volume in cc Calculated from the 3 Heartbeats Shown in Fig. 3. (Heart rate 120.)

Heartbeat No.	1	2	3
Forward flow during cardiac ejection	8.67	7.67	7.86
Backward flow following ejection, marked x	.68	.64	.71
Forward flow during diastole, marked y	.02	.01	.13

noted that backflow amounted to 9% of the stroke volume. In the other experiments this value was smaller. The tracing in Fig. 3, which exhibits large backflows, was primarily selected in order to illustrate the suitability of the flowmeter to record backflow.

Fig. 4 shows a segment of an original record illustrating the action of the "zeroing" device. This record was taken from an experiment in which the chest was closed and zero flow was obtained by actuating the zeroing cylinder (G in Fig. 1) through the cable release from the outside of the animal. Pulmonary artery flow during the first cardiac cycle showed a pattern similar to that described in Fig. 3. However, it is to be noted that during atrial systole (marked AS) flow in the pulmonary artery was briefly accelerated and decelerated. This is obviously due to an impact from the neighboring atria on the proximal region of the pulmonary artery, which caused the forward and backward movement of the fluid column. The impact of atrial systole is also seen in the pulmonary artery pressure tracing and to a mild degree even in the aortic pressure pulse contour. Atrial impacts on arterial pressure curves have often been described; however, those on flow have not. One must be cautious, therefore, in assuming that blood does not flow in the pulmonary artery trunk during diastole. Baxter and Pearse(6) based the determination of "zero flow" in the pulmonary artery on this assumption.

During late systolic ejection of the second heart beat (marked x in Fig. 4) the zeroing cylinder was pushed forward. This maneuver took 80 milliseconds, judged from the duration of the impact during late systole on the pulmonary artery pressure tracing (marked y). The absence of turbulence or oscillations in the flowtracing during the cylinder movement at the end of the ejection period should be noted. From then on, the flow record is practically a straight line. The backflow after the ejection did not register, nor did the forward flow during the next ejection. However, there was slight bristle movement during the ejection, which was presumably caused by some turbulence of the blood rushing past the cylinder opening. The pressure tracings in the third heart cycle remained, of course, unaffected by the zeroing cylinder. From these findings it must be concluded that the cylinder affords an effective means of protecting the bristle from the streaming blood and thus for establishing zero flow during the experiment without interfering with the major blood flow in the vessel.

One of the important conditions for all measurements of volume flow is the constancy of the vessel's cross-sectional area. This is usually accomplished by cannulating the vessel with a cannula of fixed diameter. Since in these experiments the cross-sectional area of the pulmonary artery was kept constant by an external metal band it was felt that the flow pattern should be checked against that of a

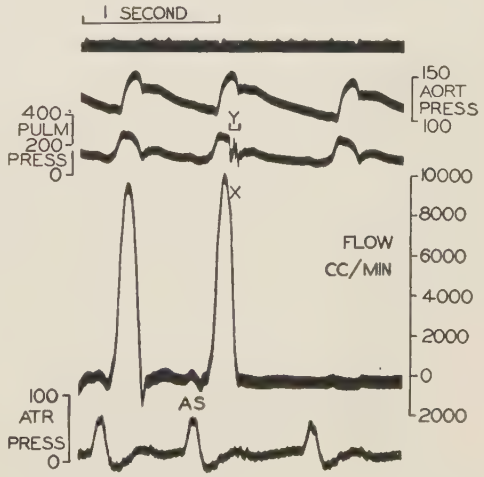

FIG. 4. Cardiac output phasically recorded with the bristle flowmeter illustrated in Fig. 1 and 2, showing effect of zeroing cylinder on flowtracing. (Dog, 28 kg weight, heart rate 98, cardiac output 2156 cc.) Tracings from top to bottom: Time, aortic pressure in mm Hg, pulmonary artery pressure in mm H_2O. Flow in pulmonary artery trunk in cc/min., right atrial pressure in mm H_2O. (¼ of original.)

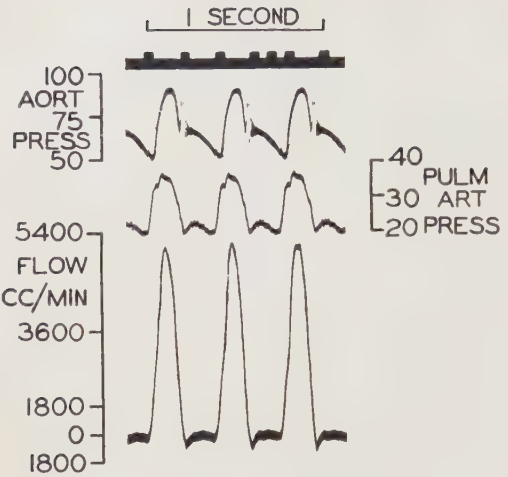

FIG. 5. Cardiac output phasically recorded with conventional bristle flowmeter(1-3) which fixes the cross-sectional area of the pulmonary artery trunk by the insertion of a ring-like cannula head into lumen of vessel. (Cannula internal diam. 12 mm, heart rate 155, dog 15 kg weight, cardiac output 1240 cc.) Tracings from top to bottom: time, aortic pressure in mm Hg, pulmonary artery pressure in mm Hg, flow in pulmonary artery in cc/min. (⅓ of original.)

flowmeter of conventional design. Therefore, in addition to the 10 experiments, 3 others were undertaken in smaller dogs in which the ring-like head of a standard bristle flowmeter (1-3) was quickly inserted through a small incision into the pulmonary artery trunk. This was done without occlusion of the artery but with loss of a considerable amount of blood. Fig. 5 shows a segment of a record illustrating the pulmonary artery flow pattern when the blood passed through the cannula head of the flowmeter, which fixed the inside diameter of the vessel. It is noted that the profile of the flow curve is very similar to those shown in Fig. 3 and 4.

Discussion. The modification of the bristle flowmeter described here has been successfully employed in our laboratory for one year for various cardiac output studies. Its application is not limited to right heart output measurements, since the device can be easily inserted into other major vessels such as the aorta. Its use is particularly indicated in all investigations where accurate knowledge of phasic changes of right heart outflow is indispensable, where stroke for stroke changes occur and where the existence of an "unsteady state" does not permit the application of indirect methods such as the Fick determination.

The advantages of the new method are the following: 1. The flowmeter can be inserted at leisure into the pulmonary trunk without blood loss and without interrupting flow. 2. The resistance to blood flow introduced by the flowmeter is negligible. 3. Accurate zero flow can be determined at any time during the recordings without vessel occlusion or interference with the circulation. 4. Changes in forward and backward flow can be faithfully recorded owing to the high fidelity of the flowmeter (125 cycles/sec.). 5. Mean cardiac output can be recorded through electrical integration by merely turning a dial at the amplifier. 6. Since the "electrical mean" averages all actual forward and backward flow at the site of the flowmeter it records what is conventionally defined as "cardiac output." 7. The sensitivity of the instrument is very high: 20 mm deflection of the galvanometer 50 cm in front of the camera for a flow of 20 cc per second when the electrical signal is amplified only 50 times. 8. The insertion of the cannula induces a negligible amount of trauma and furthermore the animals remain in good condition for many hours.

Baxter and Pearse(6) reported recently a differential pressure method with which they recorded pulmonary artery flow in anesthetized cats. Their method suffered, however, from the following shortcomings which were avoided in the new method described here. 1. The pulmonary artery trunk must be briefly occluded for the insertion of the flowmeter, which damages the right myocardium by overdistention in normovolemic animals. 2. The device introduced into the artery offers resistance to flow, and this may distort the pattern of phasic flow(7). 3. The determination of zero flow, upon which the accuracy of all flow measurements depends, is unreliable. It is accomplished by setting automatically an electronic "zero" at flow during diastole. According to Baxter and Pearse, this is done: "under the assumption that diastolic flow in the trunk of the pulmonary artery is negligible, but occasionally there was evidence of slight flow at the beginning of diastole. This

slow changing forward flow tended to upset the tuning of the circuit and the measurement of subsequent beat outputs." 4. The automatic zeroing precludes the recording of backflow and analysis of flow changes during the cardiac cycle, particularly with valvular lesions. 5. The low sensitivity of 1 mm deflection for 3.6 cc per second limits the accuracy of the flow determinations.

Summary. A modification of a bristle flowmeter is described which permits direct recording of cardiac output and its fluctuations from one heartbeat to another. The device is inserted into the trunk of the pulmonary artery of anesthetized, heparinized dogs without blood loss and without interrupting blood flow. Zero flow can be established by mechanical means without occluding the vessel. Phasic changes during the cardiac cycle consist of a marked forward flow during cardiac ejection and a brief period of backflow following ejection. Mean cardiac output can be directly recorded by electrically averaging the phasic flows.

The authors wish to thank Mr. Willard Stires for the cannula construction, Dr. Julius Praglin for his help in electronic designs and Dr. F. L. Clement and Mr. Clyde Bratton for their assistance.

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Effect of a Mercurial Diuretic on Thyroid Uptake and Renal Clearance of Radioiodine.* (21135)

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This study was undertaken to determine what effect, if any, the administration of a mercurial diuretic exerted on the validity of the radioiodine uptake procedure as a measure of thyroid function. It has been demonstrated by Meyers(1) that the analytic procedure for the determination of serum protein-precipitable iodine is rendered non-valid by the recent administration of meralluride sodium. Because of the competition between the thyroid and the kidney for the available supply of inorganic iodide, it would also appear that should mercurial diuresis significantly alter renal iodide excretion, this might adversely influence the validity of thyroid radioiodine uptake studies. The effect on other halogens is better known: the production of a chloride diuresis is one of the early effects of mercurial diuretics; a similar effect has been found in the experimental animal for the bromide ion(2). In man, the renal clearance of iodide, unlike that of bromide, has been stated to appear independent of variations in chloride intake and excretion(3); in the dog, however, the renal clearance of iodide, like bromide, is largely determined by the rate of chloride excretion(4).

Method. In this study, thyroid radioiodine uptakes were initially determined at 6 and 24 hour periods on 8 euthyroid patients, 3 of whom had congestive heart failure. Uptakes were repeated 2 weeks later, at which time the intramuscular administration of 2 ml

* The author wishes to express his deep appreciation to Dr. Franz K. Bauer for his guidance in this study.

TABLE I. Renal Clearances before and after Injection of 2 ml Meralluride Sodium.

Patient	Period	Urine vol, ml	Chloride excretion, meq	I^{131} clearance, ml/m
NT				
Before Hg	A	18		47.2
	B	18	2.00	46.0
After	C	100		48.9
	D	40	5.05	43.6
FD				
Before	A	115		22.0
	B	140	5.65	24.6
After	C	230		23.4
	D	175	15.35	18.4
HT				
Before	A	160		61.0
	B	125	10.16	44.5
After	C	155		42.4
	D	190	36.90	49.0
HF				
Before	A	350		28.8
	B	320	4.85	27.8
After	C	90		28.4
	D	65	17.91	25.9

meralluride sodium preceded the I^{131} test dose. In addition, renal clearances of radioiodine were determined on 4 patients before and after intramuscular administration of 2 ml of meralluride sodium. All had clinical diagnoses of heart disease without congestive failure. Three were euthyroid; one was slightly hypothyroid. Each patient received orally 40 microcuries of I^{131} along with an initial oral tap water load of 500 to 1500 ml. All studies were conducted during the 6-hour period following oral administration, with the assumption that the protein bound I^{131} fraction during this period was insignificant. Clearances were determined for two 30-minute intervals (Table I, Periods A and B) during the third hour following administration; 2 ml meralluride sodium were given intramuscularly at the end of third hour; and two 30-minute clearances (Periods C and D) were again determined during third hour following the mercurial injection. Clearances were calculated by the standard formula of total urinary I^{131} excretion during the 30-minute period divided by the plasma level at the mid-point of each period—a procedure shown by Berkson(5) to agree generally with the more exact parametric method of calculation of clearance after one-dose administration. All specimens were counted in a deep well type scintillation counter.

Results. Thyroid Uptake. Of the 8 patients who had standard 6 and 24 hour thyroid uptake studies, there was no significant difference, outside of the limits of reproducibility of the procedure, in determinations with and without the simultaneous administration of 2 ml meralluride sodium.

Renal Clearance. The results in the 4 patients with renal clearance studies are summarized in Table I. The extent of volume diuresis varied widely during the test periods, apparently depending upon the relative diuretic effect of the water load which preceded the two control clearance periods and that of the mercurial injection which preceded the last 2 clearance periods. Both chloride and radioiodide excretion remained remarkably independent of the fluctuations in degree of water diuresis. In all patients, chloride excretion during the test period following mercurial injection rose to between 250% to 350% of the control values. In contrast, during the same period renal clearances of radioactive iodine were essentially unchanged, remaining within the range of 88% to 98% of the control values. These effects are summarized graphically in Fig. 1, in which response to meralluride sodium injection is compared to the control value, represented as 100%.

Comment. These findings suggest an almost total indifference of the renal mechanism for excretion of iodide to the alterations in renal tubular function associated with the early effect of a mercurial diuretic. This lack of re-

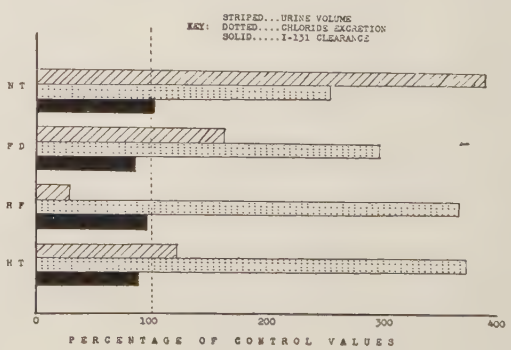

FIG. 1.

sponsiveness is in marked contrast to the effect on chloride excretion. In application, the results also suggest that the validity of the standard radioactive iodide uptake studies of thyroid function is not affected by the administration of a mercurial diuretic.

Summary. 1. Meralluride sodium (Mercuhydrin®) had no effect upon the results of the 6 and 24 hour thyroid uptake procedure in 8 euthyroid patients. 2. Renal I^{131} clearances were determined before and after injection of meralluride sodium in 4 patients, and compared with simultaneous chloride excretion data. Radioiodide clearances remained unchanged in the face of a consistent and marked increase in chloride excretion.

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Effect of Colchicine on Eosinophil Count in the Hypophysectomized Rat.* (21136)

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In previous clinical studies(1) it has been observed that 3 mg of colchicine injected intravenously has an eosinopenic effect. On the other hand, it has been shown by various authors(2-4) as well as by our own investigations, that there is no demonstrable effect on the excretion of the urinary corticoids following colchicine administered orally or parenterally. Thus it appeared possible that this eosinopenic response to colchicine was not mediated through the pituitary-adrenal relationship. The purpose of this study has been to determine whether colchicine administration causes eosinopenia both in normal and in hypophysectomized rats.

Methods. The experiment was run with 22 young male rats of the Wistar strain. Eleven were hypophysectomized. Six of the hypophysectomized and 7 of the normal rats were injected subcutaneously with 1 ml of colchicine* in physiological saline solution in a concentration of 0.2 mg per ml. Control groups were similarly injected with 1 ml of physiological saline solution. For counting the eosinophils we employed the staining method of Speirs and Meyer(5). The stain contained 10 ml phloxine solution (1%), 1 ml diethylene glycol, 4 drops of alconox (0.5%), and 30 ml distilled water. To this solution 8 ml of acetone were added just before use. The eosinophils were counted before injecting colchicine or saline, and were again counted

TABLE I. Eosinophil Response to Colchicine and to Saline Injections in Intact Rats.

	Eosinophils/mm³ following No. of hr		
	0	4	48
	Saline inj.		
	182	200	500
	225	25	113
	225	138	578
	273	100	617
Mean value	226	116	452
	Colchicine inj.		
	475	38	250
	317	38	38
	13	13	100
	75	163	113
	100	57	113
	88	138	63
	1125	210	125
Mean value	313	94	115

* The colchicine used in these experiments was kindly provided by Dr. Glenn Ullyot, Smith, Kline and French Laboratories, Philadelphia.

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‡ Paper No. 362.

TABLE II. Eosinophil Response to Colchicine and to Saline Injections in Hypophysectomized Rats.

	Eosinophils/mm³ following No. of hr		
	0	4	24
		Saline inj.	
	140	Blood clotted	80
	168	325	200
	400	450	400
	350	385	125
	125	550	150
Mean value	237	427	191
		Colchicine inj.	
	225	125	—
	425	275	—
	140	25	—
	340	125	—
	150	125	—
	140	25	—
Mean value	237	117	

4 and 24 to 48 hours after the injections. Two series of animals were studied on different days but at the same hours of the day.

Results. In Table I are reported the results of injection of colchicine, 1 mg/kg in normal rats. The fall in eosinophil count after injection of colchicine is striking, with a difference of the means of minus 70%. The number of eosinophils continued low after 48 hours.

Intact rats injected with saline showed a decrease of 49%. This eosinopenic response to the stress of handling and tail clipping is well known, and is in agreement with the results of Speirs and Meyer(5).

The data of Table II show a 50% decrease in the number of circulating eosinophils in hypophysectomized animals 4 hours following the injection of colchicine (1.6 mg/kg of rat). All the hypophysectomized animals died from this dose of colchicine between 4 and 24 hours after injection. Since all animals were given 1 ml of the colchicine solution, the hypophysectomized rats received a relatively higher dose than did the intact rats because of their lesser body weight. We have noticed only a slight difference in the values of the initial eosinophil counts between hypophysectomized and normal intact male rats.

In hypophysectomized rats injected as controls with 1 ml saline, the eosinophils increased to an average of 81% above pre-treatment levels.

Discussion. The results of these studies show that the injection of colchicine is followed by a decrease in eosinophil count of 50% in hypophysectomized and 70% in normal intact male rats. It was interesting, on the other hand, to observe an increase of 81% in the eosinophil counts of hypophysectomized rats following saline injection in contrast to a decrease of 49% in normal intact rats. The decrease in eosinophil counts in the normal intact male rats following injection of either colchicine or saline is thus in contrast to the effect of these 2 injections in the hypophysectomized rats. The decrease in eosinophils following colchicine administration in the hypophysectomized rats suggests that the presence of the hypophysis is not essential to this pharmacological phenomenon. It is, perhaps, of even more interest to observe that the greater decrease in eosinophils following colchicine occurred in intact animals which, due to their relatively greater body weight, received 1.0 mg instead of 1.6 mg/kg of body weight.

Since, in these experiments, a 50% decrease in the circulating eosinophils occurred in hypophysectomized rats, it is evident that the decrease of eosinophils in the rat is not necessarily mediated by the pituitary gland. The eosinopenic effect of colchicine in the hypophysectomized male rat may be due to direct adrenal stimulation or to a peripheral effect not involving the pituitary-adrenal interrelationship. To clarify this question similar studies are being performed in adrenalectomized animals.

Summary. 1. Colchicine injected into normal male rats caused a 70% decrease in circulating eosinophils. Hypophysectomized rats similarly injected with colchicine showed a 50% diminution. 2. Saline injections in normal rats caused a 49% decrease in eosinophils, while in hypophysectomized rats there was an increase of 81%. 3. The eosinopenic response to colchicine appears, in the main, to be due to a direct adrenal stimulation or to a direct peripheral effect.

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Blood Clotting Time and Tissue Mast Cell Number of the Bat (*Myotis lucifugus*) in Different Physiological States. (21137)

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The bat is one of a number of mammals which in nature pass the winter in a state of dormancy characterized by low rates of respiration, heart beat, blood flow and oxygen consumption(1). Laboratory studies have shown that the resting bat is essentially poikilothermic(2,3) and that its rates of blood flow(3) and oxygen consumption(2,3) vary directly with the ambient temperature. At low temperatures (5-10°C) the red blood cells traverse the vessels in clumps and rouleaux, but thrombi and stoppage of flow are not seen(3). At high temperatures (20°C and upward) blood flow is much like that found in other mammals. Since intravascular clotting and thrombus formation do not accompany the sluggish circulation of the dormant bat, we have tried to establish whether different blood clotting times characterize the profoundly different physiological states which may be induced in the bat. We have already noted(4) that clotting time is markedly prolonged when the summer bat is exposed to cold. The present paper is concerned with the extension of these studies to winter bats and with the possibility that changes in clotting time might be accompanied by changes in the tissue mast cells which are thought to store and release the anti-coagulant, heparin(5,6).

Methods. *Small brown bats* (*Myotis lucifugus*) were collected in the winter from a cave in northern Illinois and in the summer from the attic of a farm house in southern Indiana. Blood clotting times were determined (in all cases at 23°C) by the capillary tube technic on samples obtained by puncturing a large vein in the web between the hind legs and from the cut carotid artery (the clotting time of the blood from each source was essentially the same). In each bat counts were made of the number of mast cells in single 12 μ thick sections of duodenum magnified 264x. The tissues were fixed in absolute alcohol and stained with toluidine blue. Studies were carried out at the time of collection and later in the laboratory at various times after placing summer bats in a refrigerator (5°C) and winter bats in an air conditioned room (23°C).

Results and discussion. In nature markedly different clotting times distinguish the summer bat from the winter bat (Table I). That of the former is short as in the homoiothermic mammals, while that of the latter is very long as in the aestivating ground squirrel(7) and in the hibernating hedgehog(8) and hamster(9). We interpret these data to indicate the existence of an adaptive mechanism which (a) prevents thrombus formation under the conditions of slow blood flow found in the dormant winter bat and (b) allows for rapid blood coagulation in the active summer bat. Table I also indicates that such a mechanism operates upon exposure of summer and winter bats to different ambient temperatures. Summer bats become dormant upon exposure to cold and show a marked prolongation of clotting time, while dormant winter bats awaken upon exposure to 23°C and manifest

TABLE I. Blood Clotting Time and Tissue Mast Cell Number of the Bat in Different Physiological States.

Experimental conditions	No. of bats	Blood clotting time (min.) Min	Max	Avg	Mast cell No. (avg)
Summer bat (on collection)	10	1.5	24	7.4	121
Winter bat (" ")	13	45	>360	(180)*	119
Summer bat					
On 16th day at 5°C	4	72	275	149	152
" 50th " " 5°C	2	170	210	190	169
Winter bat					
After 60 min. at 23°C	4	2	11	5	—
On 1st day at 23°C	9	5	26	12	50
" 4th " " "	10	7	32	18	26
" 7th " " "	10	6	60	25	19
" 14th " " "	10	6	47	19	61
" 21st " " "	6	4	13	8	40

* Median.

a sharp decrease in clotting time. The mechanism is quickly called into play, since within an hour after bringing the dormant winter bat into a warm environment (23°C), the clotting time is reduced to that of the summer bat.

The mast cell data are also found in Table I. The tissue mast cell population of sections of duodenum is the same in the dormant winter bat as in the active summer bat. Upon exposure of the former animal to 23°C and the latter to 5°C there appear to be decreases and increases respectively in the mast cell content of the duodenum. The reduction of mast cells in the winter bat is statistically significant, while the increase in the summer bat is not. Since these changes and indications of changes in mast cell number are accompanied by alterations in the clotting time of the blood, it is possible that the mast cell alterations, in part at least, may account for the different clotting times. Thus increased numbers of mast cells are associated with an increased availability of heparin and prolonged blood clotting; while decreases in mast cells are associated with a lowered availability of heparin and rapid blood clotting. Similar changes have been observed in the hedgehog and have been given a like interpretation (8). Microscopic examinations of sections of the duodenum and of whole mounts of the skin gave no information on the fate of mast cells disappearing when the winter bat is placed at 23°C. The number of abnormal (degenerated) (10) mast cells was small and essentially constant in all the conditions studied. Failure to find differences in the absolute number of mast cells in duodenal sections from summer and winter bats might be accounted for by differences in strain.

Summary. 1. Blood coagulation times and tissue mast cell counts were determined on summer and winter bats and upon their exposure to 5°C and 23°C, respectively. 2. In nature the blood clotting time of the active summer bat is short, while that of the dormant winter bat is prolonged. Exposure of the summer bat to 5°C and the winter bat to 23°C in the laboratory results in a lengthening and a shortening of the clotting, respectively. The number of tissue mast cells in the duodenum appear to increase in the former case and decrease in the latter instance. 3. The above changes are interpreted as indicating the presence of a mechanism which prevents thrombus formation under the conditions of slow blood flow during dormancy and which allows for rapid clotting when the bat is active. The mast cell changes suggest that the alterations in clotting time are determined at least in part by alterations in the availability of heparin to the circulation.

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Inhibition of *in vitro* Heme Synthesis from N^{15}-Glycine by 2-Ethyl-5-methylbenzimidazole.*† (21138)

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Avian erythrocytes incorporate N^{15} into heme when incubated with N^{15}-glycine and provide an *in vitro* biological system for the study of heme synthesis(1). Because of the chemical relationships of methyl-substituted benzimidazoles to the benzimidazole moiety of vit. B_{12} and to the purines, we investigated the effect of their addition to this system(2).

2,5-Dimethylbenzimidazole previously had been noted to act as a growth depressant when fed to rats in tests of compounds for vit. B_{12}-like activity(3). It inhibited red blood cell formation in mice made anemic by phenylhydrazine injection(4) and inhibited influenza virus multiplication(5).

We found that 2,5-dimethylbenzimidazole and 5,6-dimethylbenzimidazole prevented incorporation of N^{15} from N^{15}-glycine into heme. Benzimidazole and 2-methylbenzimidazole at the same concentration had slight but not comparable activity. 5-Methylbenzimidazole had an inhibitory effect intermediate between that of benzimidazole and 2,5-dimethylbenzimidazole(2).

These compounds, in approximately the same concentration and in precisely the same relative order of inhibitory activity, were found by Tamm *et al.*(6) to inhibit multiplication of influenza A or B virus. The striking coincidence of relative inhibitory activities of the methyl-substituted benzimidazoles for 2 widely different systems, *in vitro* hemoglobin synthesis by avian erythrocytes and virus duplication, indicated to us that some fundamental mechanism underlying both processes might be involved.

Tamm *et al.*(6) found 2-ethyl-5-methylbenzimidazole to be a much more potent antiviral agent than any of the benzimidazoles studied by us in the avian erythrocyte system(2). It was considered to be approximately 7 times more effective than 2,5-dimethylbenzimidazole and 19 times more so than benzimidazole. It is thus of importance to know whether 2-ethyl-5-methylbenzimidazole likewise is a more effective inhibitor than 2,5-dimethylbenzimidazole for the synthesis of heme by the avian erythrocyte. This has been determined to be so in the present study and supplies additional support to the concept that the same fundamental mechanism underlies both processes.

Experimental. Fresh chicken blood was obtained for each experiment. Penicillin and streptomycin were added to inhibit microbial action during incubation(1). Twenty ml blood samples were incubated with the experimental compound and 25 mg N^{15}-glycine (31 atom % N^{15}) in 50 ml Erlenmeyer flasks with constant shaking for 24 hours in a water bath at 37°C. In each experiment the effect on

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TABLE I. Effect of 2-Ethyl-5-methylbenzimidazole and 2,5-Dimethylbenzimidazole on Incorporation of N^{15} from N^{15}-Glycine into Heme by Erythrocytes of Chicken Blood during *In Vitro* Incubation.

Exp.	N^{15} conc. in hemin* Control	Test		Supplement†
	Atom % excess		Molar conc.	
1	.066	.054	.0017	5 mg 2,5-dimethylbenzimidazole
		.022	.0016	5 " 2-ethyl-5-methylbenzimidazole
2	.080	.034	.0034	10 " 2,5-dimethylbenzimidazole
		.003	.0031	10 " 2-ethyl-5-methylbenzimidazole
3	.081	.008	.0068	20 " 2,5-dimethylbenzimidazole
		.051	.0009	3 " 2-ethyl-5-methylbenzimidazole
4	.078	.046	.0006	2 " 2-ethyl-5-methylbenzimidazole
		.042	.0085	20 " benzimidazole

* Hemin isolated from chicken blood has slightly increased N^{15} concentration over tank nitrogen similar to that of hemin from normal dog and human blood(9). Values on the order of .002 to .006 atom % excess thus indicate complete inhibition of N^{15} incorporation into heme.

† These were prepared by refluxing the appropriate diamine and acid according to the general procedure of Wagner and Millett(10). The compounds were recrystallized several times, and melting points and nitrogen analyses were checked before use. Benzimidazole and 2,5-dimethylbenzimidazole have been described previously. 2-Ethyl-5-methylbenzimidazole melted at 168°-169°C, and gave the following nitrogen analyses: 17.5%, 17.4%, 17.4% (theoretical 17.5%).

heme synthesis of the substance studied was determined by comparison with a control sample of the same blood incubated with N^{15}-glycine alone at the same time under identical conditions. Three samples of the same blood were incubated at the same time, 2 test samples with one control.

Hemin was isolated as described by Shemin and Rittenberg(7) and recrystallized according to Fischer(8). The N^{15} atom percent excess over tank nitrogen was determined on the nitrogen gas from duplicate samples of hemin isolated from each incubated blood. Previous experiments had demonstrated that the same blood incubated with N^{15}-glycine under identical conditions yielded hemin with the same N^{15}-content(2).

Results. Table I shows that 2-ethyl-5-methylbenzimidazole was considerably more inhibitory than 2,5-dimethylbenzimidazole. Thus 5 mg (.0017 M), which produced minimal (18%) inhibition with 2,5-dimethylbenzimidazole, gave marked (67%) inhibition with the 2-ethyl-5-methyl derivative (Exp. 1). Ten mg 2-ethyl-5-methylbenzimidazole (.003 M) gave complete inhibition, whereas this required 20 mg 2,5-dimethylbenzimidazole (Exp. 2 and 3). Two mg 2-ethyl-5-methylbenzimidazole (.0006 M) produced approximately the same degree of inhibition as 20 mg benzimidazole (.0085 M). Thus, in Exp. 4, .0006 M 2-ethyl-5-methylbenzimidazole was as effective as 14 times the concentration of benzimidazole, and at the .0016 M level the percentage decrease in N^{15} uptake from the control experiment was about 4 times greater with the 2-ethyl-5-methyl derivative than with 2,5-dimethylbenzimidazole. Complete inhibition occurred with half the amount required with 2,5-dimethylbenzimidazole (Exp. 2).

Discussion. Demonstration that 2-ethyl-5-methylbenzimidazole is considerably more inhibitory than 2,5-dimethylbenzimidazole for synthesis of heme by chicken erythrocytes incubated *in vitro,* similar to findings of Tamm *et al.*(6) in studies on the inhibition of influenza virus multiplication, adds further support to our belief that a fundamental mechanism underlying both processes is involved. This might be the result of selective inhibition of a basic step in the mechanisms whereby nucleic acid (or nucleoprotein) plays some very important role in biosynthesis. It is of interest to note that the 5 (or 6) position of benzimidazole is analogous to the 2 position of the purines. Recent discovery of 2-methyladenine in biological material(11) brings attention for the first time to the existence of analogous methyl-substituted purines in nature.

Summary. 2-Ethyl-5-methylbenzimidazole was found to be considerably more inhibitory than 2,5-dimethylbenzimidazole, or benzimidazole, for the synthesis of heme by chicken erythrocytes incubated *in vitro*. This was in the same relative order of inhibitory activities as was noted for multiplication of influenza A or B virus, and is considered to add further support to the concept that the same basic mechanism, possibly involving a fundamental role of nucleic acid in biosynthesis, underlies both processes.

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Progesterone-Like Activity in the Plasma of Ovoviviparous Snakes.*† (21139)

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Studies on the natural occurrence of progestin have been carried out primarily on mammals. This hormone has been found in a large number of mammalian species and seems to be ubiquitous in this class. The available evidence indicates that the hormone is produced in the mammal by the corpus luteum (and to a lesser extent, the pre-ovulatory follicle), the placenta, the adrenal cortex and possibly the testis. Fraps, Hooker and Forbes(1,2) reported that substances with progesterone-like activity were found in the plasma of ovulating hens, non-ovulating hens, and cocks, but not in the plasma of capons. Porto(3) reported the presence of a substance in an extract of 158 corpora lutea of two species of ovoviviparous South American snakes, *Bothrops jararaca* and *Crotalus terrificus*, that gave a 2+ endometrial reaction in the McPhail assay for progesterone(4). This is the only evidence thus far for the presence of substances with progestational activity in reptiles. It was therefore felt worthwhile to examine the blood plasma of snakes by using the sensitive bioassay method for small amounts of progesterone described by Hooker and Forbes(5). Such information might conceivably lead to a better understanding of the evolutionary significance of this hormone and its role in the lower vertebrates.

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Materials and methods. Preliminary experiments indicated the presence of substances with progesterone-like activity in the plasma of 2 pregnant water snakes, *Natrix s. sipedon.* These observations were confirmed and a quantitative study was made of the hormone content of the plasma. The animals used in these experiments were predominantly water snakes, *Natrix s. sipedon,* from Thurmont, Md., while a few observations were made on garter snakes, *Thamnophis radix,* collected in Champaign-Urbana, Ill.

Blood was obtained by cardiac puncture with a No. 18 or 20 short-beveled needle. The location of the heart was readily detected by its pulsations against the ventral body wall at scutes 25 to 30. Since the free edges of the scutes are directed caudally, the needle was inserted under the edge of one of the scutes posterior to the site of the cardiac impulse and run obliquely forward to reach the heart. The blood was treated with heparin or citrate and the plasma removed and stored at –20°C. All assays for progesterone-like activity were carried out according to the Hooker-Forbes technic(5) which has been shown to be valid for the assay of untreated serum or plasma(6). Although the identity of the substance assayed by the Hooker-Forbes test is unknown, in keeping with the previous studies, the activity of the progestin has been standardized against progesterone and will be expressed as μg equivalents of progesterone.

Experimental. Cieslak(7) states that in *Thamnophis radix* the ovarian follicles fall into 3 macroscopically distinguishable size groups. This situation has been found to obtain for *Thamnophis sirtalis* and *Natrix sipedon* and may be true for snakes in general. A new crop of follicles appears during the early summer. These undergo a long period of slow and gradual growth which culminates in their third spring with a vigorous preovulatory growth spurt. During late April and May, in the northeastern states, the ova of the largest size group increase rapidly in volume by the deposition of large quantities of yolk. Ovulation usually occurs in late May or in June. In Table I, the snakes designated as having inactive ovaries had only small follicles of the immature type. Those listed as preovulatory had ripening follicles in which appreciable amounts of yolk had already been deposited.

TABLE I. Concentration of Progesterone-Like Activity in the Plasma of Ovoviviparous Snakes.

Snake No.	Status of snake	Progesterone-like activity,† μg/ml
NM-25-52	Inactive ovaries	.3
NMT-18	Inactive ovaries with atretic follicles	1.0
NM5-52	Preovulatory	.3
NMAB-52	"	.3
NM12-52	Early ovulation	2.0
NI-1-52	Pregnant, ⅓ development*	4.0
NM26-52	" ⅓ " *	4.0
NM29R	" ⅔ " *	4.0
NMAL	" ⅔ " *	6.0
T1-11	" full term*	8.0

* Refers to the degree of development seen in the fetuses.

† Expressed in μg equivalent of progesterone.

It is extremely difficult to establish a known date of ovulation in these species. Females brought into the laboratory prior to the time the follicles are fully ripe often show some interference with ovulation. There may occur a complete failure to ovulate, a delay in ovulation or ovulation of only a portion of the maturing ova, the remainder undergoing follicular atresia. In two studies(7,8) ovulation has been found to occur in a regional population of garter snakes over a period of about three weeks. The gestation period is roughly 9 to 10 weeks. Thus an animal that ovulated early could be at full term at a time when an animal which had ovulated late was at two-thirds term. In this study, the estimation of the stage of pregnancy was made on the degree of development of the embryos and the amount of yolk remaining in each uterine ovum at the time of laparotomy or autopsy.

The plasma of snakes with preovulatory follicles or inactive ovaries contained activity equivalent to 0.3 to 1.0 μg of progesterone per ml. Following ovulation the concentration rose to 2 μg and then showed a progressive increase during pregnancy. The highest titer (8 μg per ml) was obtained at full term. This was seen in a snake which at autopsy in August contained 4 fully developed living fetuses, 3 dead but fully developed fetuses

and 7 ova in which there had been no development or in which cessation of development had occurred early in pregnancy (Table I).

The plasma of 2 male snakes with fully developed testes (June 12 and 29) showed a level equivalent to 0.3 and 1.0 μg progesterone per ml.

Discussion. The presence of a substance in the blood plasma of snakes that is capable of eliciting a reaction that is thought to be specific for progesterone or a substance very similar to progesterone raises several questions of importance to comparative endocrinology. A problem that must remain for future research is that of the chemical nature of the active agent. Yet, regardless of what it may prove to be, its physiological importance in snakes is probably quite different from that of progesterone in mammals. Bragdon(8) has shown both in the garter snake and water snake that the corpora lutea are not essential for the maintenance of pregnancy, nor does abortion occur following hypophysectomy. However, the increase in concentration of the hormone in the plasma during gestation suggests a progestational function. This in some respects is similar to the condition in the ewe (9) and rabbit(10) in which progesterone increases in the blood during pregnancy and in these animals is indispensable for a successful gestation.

Although the hormone in snake blood produces a progestional reaction in the uterus of mice, the available experimental evidence indicates that if it has a function in the snake, it is one concerned with some other physiological process than gestation(8). The fact that it is also found in the blood of male snakes tends to support this supposition and at the same time raises the question as to its probable source of origin. Beall and Reichstein (11) have isolated progesterone from the adrenal glands of cattle and progestational activity has been found in the blood of cocks (2) and in males of a variety of species ranging from amphibians to human beings(12). With regard to the ovary, it is known that during the menstrual cycle in primates, progesterone appears several days before ovulation and formation of a corpus luteum(13-15), a condition that probably is quite common among mammals(16). Thus it is evident that the presence of progesterone in the blood does not depend entirely upon the existence of a corpus luteum even in animals in which progesterone is of major importance for gestation. In fact, it is conceivable that substances having progestational activity, when tested in mammals, may be of frequent occurrence in chordates during follicular development and ovulation. Carlisle(17), by using the Hooker-Forbes technic, found progesterone-like activity in the blood and ovary of the ascidian, *Ciona*, 48 hours after experimentally induced ovulation, but not in the non-breeding animal. The chemical nature of these substances of sub-mammalian origin are, of course unknown, but should they prove to be progesterone, as suggested by the Hooker-Forbes reaction, knowledge of them may be of considerable importance for an understanding of the physiological adaptations that took place in the evolution of viviparity.

It would be of interest to know the extent to which substances with progestational activity occur throughout the chordates. The findings of Carlisle(17) support the concept that progesterone-like substances are present throughout the chordate phylum. It is indeed possible that these substances originally appeared as intermediary metabolites without a hormonal action referable to pregnancy. Such a concept is of immense significance from the evolutionary point of view and the presence of progestin in the plasma of the ovoviviparous snake is added evidence. It is also intriguing to note that the hormone is not only present but increases during gestation even though it may not have an essential hormonal role in pregnancy.

Summary and conclusion. Progesterone-like activity was determined in the plasma of the ovoviviparous snake by the Hooker-Forbes technic. Snakes with inactive ovaries or preovulatory follicles showed a concentration of 0.3 to 1.0 μg equivalent of progesterone per ml of plasma. This titre rose to 2 μg following ovulation, to 4 μg by mid-pregnancy and to 8 μg per ml of plasma at full term.

Minimal amounts of progesterone-like activity were found in the plasma of male snakes. The possible role of this substance in the reptile and its evolutionary significance are discussed.

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Bioautography of Biotin and Certain Related Compounds. (21140)

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The existence in culture filtrates of *Aspergillus niger* grown in the presence of added pimelic acid of a previously unrecognized component of natural material with biological activity equal to that of biotin in satisfying this growth factor requirement of *Neurospora crassa* has been described by Wright and Cresson(1). The factor was isolated from a large quantity of *Aspergillus niger* culture filtrate and identified as biotin L-sulfoxide(2,3). It was established that biotin L-sulfoxide originates from an *in vivo* oxidation of biotin rather than an *in vitro* (purely chemical) oxidation. Biotin L-sulfoxide has activity equal on a molar basis to biotin, biotin D-sulfoxide, biocytin, and desthiobiotin in satisfying the biotin requirement of *Neurospora crassa.* For a number of other biotin-requiring microorganisms thus far examined it is less active than biotin or the D-sulfoxide. Biotin L-sulfoxide has an ubiquitous occurrence and may be expected to have some function in metabolism.

During the investigations involved in the discovery, isolation, and characterization of biotin L-sulfoxide considerable bioautographic data with biotin, biotin L-sulfoxide, biotin D-sulfoxide, biocytin, oxybiotin, and desthiobiotin as well as mold filtrates were accumulated where butanol-water-acetic acid was used as the developing solvent and *Neurospora crassa* was the assay organism. These data are being published in detail for the interest they may have for those concerned with elucidating various aspects of the biochemistry of "naturally-occurring" forms of biotin.

Methods. Synthetic D-biotin, DL-desthiobiotin and DL-oxybiotin (O-heterobiotin) were obtained from Hoffmann-LaRoche, Inc., synthetic biotin D-sulfoxide and biotin L-sulfoxide(4) were kindly supplied by Dr. D. B. Melville, natural biotin L-sulfoxide was isolated from *Aspergillus niger* culture filtrate as described previously(2), biocytin was synthesized in the laboratories of the Chemical Division of Merck and Co., Inc.(5). The solvent mixture for the paper chromatograms was prepared by shaking together one part

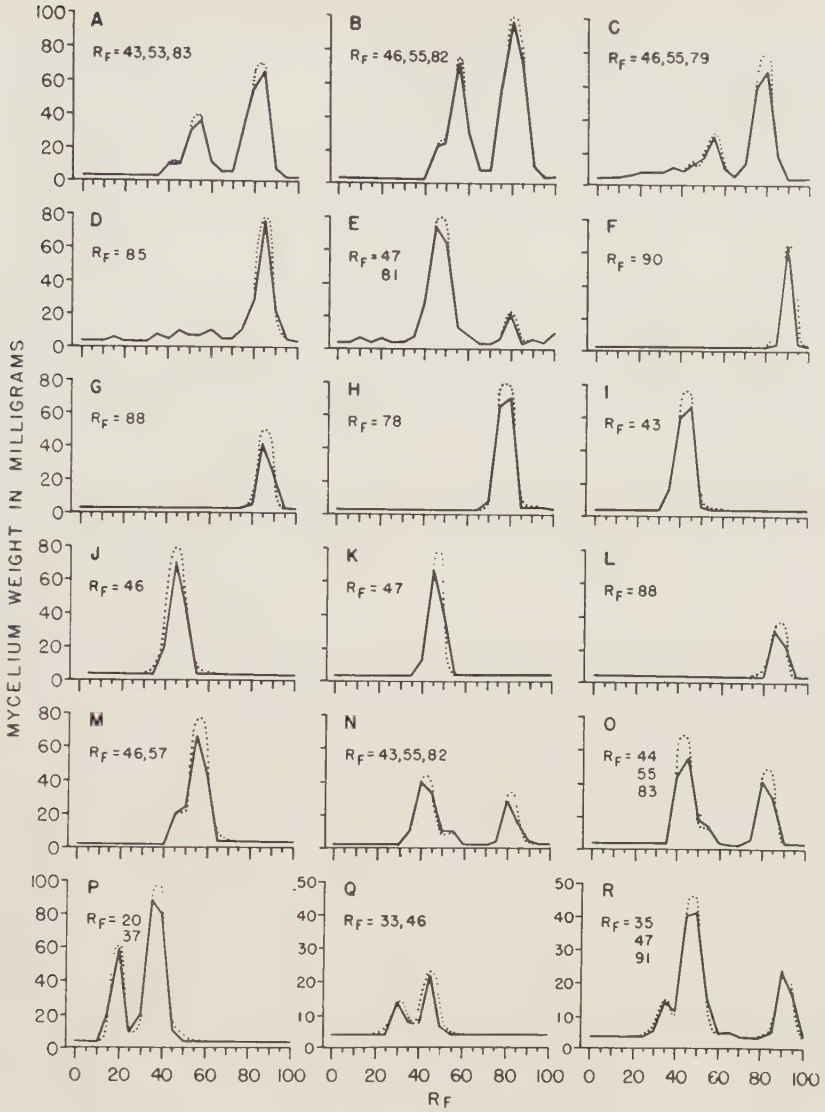

FIG. 1. Summary of bioautographic data. A, Synthetic biotin; B, synthetic biotin aerated; C, synthetic biotin shaken with *Aspergillus niger* synthetic medium; D, synthetic biotin shaken with autoclaved culture of *Aspergillus niger*; E, *Aspergillus niger* culture filtrate grown with added biotin; F, synthetic desthiobiotin; G, synthetic biotin treated with Raney nickel; H, synthetic oxybiotin; I, high potency biotin L-sulfoxide concentrate; J, synthetic biotin L-sulfoxide; K, crystalline biotin L-sulfoxide isolated from *Aspergillus niger* culture filtrate; L, high potency biotin L-sulfoxide concentrate treated with Raney nickel; M, synthetic biotin D-sulfoxide; N, high potency biotin L-sulfoxide concentrate treated with zinc and hydrochloric acid; O, high potency biotin L-sulfoxide concentrate treated with aluminum and sodium hydroxide; P, synthetic biocytin; Q, *Aspergillus niger* culture filtrate; R, *Aspergillus niger* culture filtrate grown with added pimelic acid.

glacial acetic acid, 4 parts n-butanol, and 5 parts water. The butanol phase (upper layer) was placed in the bottom of a large chromatographic jar and was the developing solvent. The aqueous phase (lower layer) was placed in a receptacle in the bottom of the jar. One-hundredth γ amounts of biotin (or its equivalent in the form of the derivative studied) in 0.01-0.02 ml of 50% ethanol were placed one inch from the edge of Whatman No. 1 filter paper sheets and allowed to dry. Development of the chromatograms was by the

ascending technic(6). Sixteen to 18 hours was the development time. Development was at room temperature which was not excessive during the period of this study. Under these conditions of paper chromatography the solvent travelled about 30 cm. After development the papers were air dried in a hood. Location of the areas of microbiological activity with *Neurospora crassa* was accomplished by cutting the paper into sections which were eluted in Erlenmeyer flasks with 25 ml of basal medium. The medium used had the following composition per liter: sucrose, 20 g; ammonium tartrate, 5 g; ammonium nitrate, 1 g; potassium dihydrogen phosphate, 1 g; magnesium sulfate (heptahydrate), 0.5 g; sodium chloride, 0.1 g; calcium chloride, 0.086 g; trace elements(7), 1 ml; p-aminobenzoic acid (PABA), 10 mg. After removal of the paper sections following a one-hour elution period the flasks were plugged, autoclaved, seeded with *Neurospora crassa*—PABA-less—and incubated at 30°C for 3-4 days. The extent of growth of the mold was determined by collecting the mycelium on a wire loop, squeezing to insipient dryness and then drying to constant weight at 80° (usually 18 hours). It was convenient to cut the paper chromatograms into 21 sections such that each section represented 0.05 of an R_F unit. When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted localization of the R_F by interpolation to within 0.02 unit.

Results and discussion. The bioautographic data for the various compounds and culture filtrates are summarized in Fig. 1 in which weight of mycelium per flask is plotted as a function of the paper section number which in turn is a function of the R_F value.

As pointed out first by Davis(8), even synthetic biotin (A) yields more than one area of microbiological activity. He observed that if biotin be paper chromatographed with butanol-acetic acid as the developing solvent and the paper then applied to a plate containing medium seeded with a biotin-requiring mutant of *Escherichia coli* (bioautography) 2 areas of activity are observed with R_F values of 0.89 and 0.58. Subsequent investigation showed that the material with R_F of 0.89 corresponds to biotin, while that with R_F 0.58 corresponds to "biotin sulfoxide". The latter apparently is produced from biotin by some component of the filter paper. With an organism such as *Neurospora crassa* which responds equally well to both sulfoxides of biotin(3) 3 areas of activity are observed corresponding to biotin, biotin D-sulfoxide, and biotin L-sulfoxide. In agreement with the results of Melville *et al.*(4) the *in vitro* oxidation of biotin, in this case presumably by a component of the filter paper rather than hydrogen peroxide, leads to the formation of the D-sulfoxide in much the larger amount. With an organism such as *Saccharomyces cerevisiae* or the *Escherichia coli* mutant of Davis which apparently responds to only the D-sulfoxide only one "sulfoxide" would appear to be formed. The solvent mixture described is the only one thus far examined that yields effective separation of the 2 sulfoxides. As indicated by the data, biotin is not oxidized by any one of a number of methods of aeration. In one instance (B) a 1 γ per ml aqueous solution was aerated by a vigorous stream of compressed air at room temperature for 18 hours. In a second experiment (C), biotin (0.05 γ/ml) was shaken (200 rpm, 30°C for 5 days) with a sterile synthetic medium used for the growth of *Aspergillus niger*(1). In this instance the pH of the medium was taken to that (pH 2.5) usually resulting from the growth of the mold on the medium. In a third experiment (D) a similar amount of biotin was shaken under similar conditions with a 5-day culture of *Aspergillus niger* which had been autoclaved to stop enzyme activity. When a similar amount of biotin was added to the medium prior to growth of *Aspergillus niger* the added biotin in the presence of the growing mold was converted to biotin L-sulfoxide (E).

Desthiobiotin, as expected, yields only one area of microbiological activity (F). Raney nickel treatment of biotin gives a product (G) with only one area of activity corresponding to desthiobiotin. Desthiobiotin and biotin are not well separated by any solvent system thus far examined.

Oxybiotin, like desthiobiotin, yields only one area of microbiological activity(H). Its

R_F value in the butanol-water-acetic acid system is only slightly less than that of biotin (0.78 vs. 0.83) so that differentiation in this system is not feasible. On the other hand, the R_F value of oxybiotin is significantly lower than that of desthiobiotin (0.78 vs. 0.89) and differentiation of these 2 compounds in this system may be accomplished.

Biotin L-sulfoxide in the form of a concentrate (I) or as the synthetic (J) or isolated crystalline material (K) migrates as an entity. There appears to be no equilibrium between it and the D-sulfoxide originating from the oxidative tendency of the filter paper. Reduction of biotin L-sulfoxide in the form of a concentrate with Raney nickel leads to material (L) that yields only one zone of activity attributable to desthiobiotin. The sample of biotin D-sulfoxide used gave 2 areas of microbiological activity (M), one corresponding to biotin L-sulfoxide. Since the D-sulfoxide is the predominant form obtained on *in vitro* oxidation and the L-sulfoxide appeared to be unaffected by the filter paper, it is suggested that the sample of the D-sulfoxide examined contained a small amount of L-sulfoxide as a contaminant. When biotin L-sulfoxide in the form of a concentrate was reduced with zinc and hydrochloric acid (N) or aluminum and sodium hydroxide (O), the product on bioautography gave 3 zones of microbiological activity. This is the expected finding if it be assumed that reduction was incomplete and the biotin formed by reduction was oxidized by a component of the filter paper to give the 2 sulfoxides in the proportions previously described.

Crystalline synthetic biocytin (ϵ-N-biotinyl-L-lysine) (P) yields more than one area of microbiological activity when bioautographed with *Neurospora crassa*. It is suggested that a component of the filter paper oxidizes biocytin in a manner analogous to that observed with biotin.

Aspergillus niger culture filtrate obtained after growth on a synthetic medium without added pimelic acid (Q) contains 2 biotin derivatives detectable by bioautography with *Neurospora crassa*. The compounds from their R_F values and microbiological activity (1) are believed to be biocytin and biotin L-sulfoxide. Note in particular the complete absence of biotin. Culture filtrate obtained after growth, with added pimelic acid (R) contains biocytin, much more biotin L-sulfoxide than present without added pimelic acid, and, in addition, biotin and/or desthiobiotin or related derivative.

It is appreciated that correspondence in R_F values in a single solvent system does not serve to establish the identity of 2 compounds. The solvent system butanol-water-acetic acid described here has been found useful in obtaining *presumptive* evidence for the existence of the biotin derivatives described because, (a) the R_F values for the various derivatives are nicely spread out permitting, for example, separation of the 2 diameric sulfoxides, and (b) the R_F values in this solvent system are essentially uninfluenced by unrelated contaminants. To obtain *conclusive* evidence for the existence of a specific biotin derivative a large number of solvent systems must be employed. R_F values of biotin derivatives in most other solvent systems studied are markedly influenced by salts and other extraneous material so that some sort of preliminary concentration as, for example, 2-way paper chromatography usually is essential.

Summary. R_F values determined bioautographically where butanol-water-acetic acid was the solvent system and *Neurospora crassa* was the indicating organism are summarized for biotin, desthiobiotin, oxybiotin, biocytin, biotin L-sulfoxide, biotin D-sulfoxide, and the microbiologically active components of *Aspergillus niger* culture filtrate.

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A Method of Insulin Bio-Assay and Its Application to Human Plasma Fractions.* (21141)

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Methods have been described for the assay of minute quantities of insulin employing hypophysectomized-alloxanized rats and mice (1,2). Perhaps the most sensitive technic reported has been that of Anderson's group(2) utilizing decline of blood glucose levels following intraperitoneal injection of test material in hypophysectomized-alloxanized mice. A stable, elevated blood glucose level was induced by prior gavage with the polysaccharide, dextrin.

Certain modifications of the technic of Anderson's group are here presented. A preliminary account of the application of this modified procedure to study of human plasma fractions is also given.

Procedure. Male, 15-20 g mice of the CAF-1 strain obtained from Jackson Memorial Laboratories, Bar Harbor, Maine, are hypophysectomized by the anterior parapharyngeal approach. Anesthesia is induced by intraperitoneal injection of Nembutal (R), 75 mg per kilo. Following surgery, the mice are retained in a closed oxygen jar until their recovery from anesthesia. Diet consists of ground Purina laboratory chow and 3-5% glucose in water *ad libitum,* supplemented by lettuce, cabbage, bread crumbs and dog meal. The mice are alloxanized 7-9 days after hypophysectomy with 0.1-0.25 ml of alloxan solution in a dosage of 70 mg per kilo. This is administered into a tail vein through a No. 27 gauge hypodermic needle. Heating the mouse in a glass jar under a light bulb for 30-60 seconds dilates the tail veins sufficiently to facilitate intravenous injection. 1.25 mg of the saline suspension of the free alcohol of Cortisone are administered subcutaneously 3-4 hours before alloxanization.‡ At least 60 hours should elapse between alloxanization and use of the mice as assay subjects.

There is a marked tendency for the mice to expire in hypoglycemic shock following hypophysectomy and this problem persists after alloxanization. One ml of 5% glucose subcutaneously should be given in an effort to abort such episodes. The addition of 1.25 mg of cortisone subcutaneously may further assist the animal's survival when hypoglycemic symptoms appear. An environmental temperature of 25°-30°C, preferably 27°C, seems to be very important for the maintenance of the animals. At best, with all precautions and the most meticulous care, there is high mortality following hypophysectomy, with the attrition rate inconstant and unpredictable. Occasionally, the mortality rate may be catastrophic. In general, animals survived 1-4 weeks after operation. Mice surviving hypophysectomy beyond 2 months, in our experience, have proved to possess intact hypophyses. All animals employed for assay were examined after death for presence of pituitary tissue. Gross examination or examination with a hand magnifying lens proved to be sufficient.

The actual testing procedure commences with simultaneous intraperitoneal injection of test material and gavage with 300 mg of dextrin (Dextrin White, Mallinckrodt) in one ml of aqueous suspension. The gavage equipment consists of a one ml tuberculin syringe and a No. 20 needle, with the point removed, to which approximately 0.5 cm of polyethylene tubing is attached. The needle and tubing must be advanced into the mouse esophagus slowly and carefully. Blood samples are collected from the mouse tail vessels in 0.02 ml amounts. The tail tip is cut with shears. The tail is previously dipped into warm tap water and dried. It should be "milked" gently. Heparin powder (Hynson, Westcott, and Dun-

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TABLE I. Insulin-Like Activity of Various Substances Measured by Comparison of Blood Glucose Increments during 30 Minutes.

Material tested	No. of determinations	Mean rise in blood glucose (mg %)	Significance of difference between means by "t" test — P value — Saline	Plasma
Saline	27	146	—	—
Insulin, 0.1 mU	4	175	—	—
" 1-2 mU	4	91	>.3	—
" 4-5 mU	8	14	<.01	—
Plasma	29	106	>.2	—
Gamma-globulin	7	202	—	—
Albumin (SPPS)	13	114	>.3	—
Globulin (PGP)	45	73	<.01	>.05; <.1

Note: P not calculated if mean rise in mg % glucose for insulin or plasma fraction greater than mean rise of saline or whole plasma.

ning, Inc.) is placed on the pipette tip to prevent blood clotting. The sample of blood is diluted in 2.38 ml of distilled water. Blood glucose levels are determined by the Nelson-Somogyi method(3,4).

Results. Earlier studies indicated that it was impossible to attain a consistent plateau of blood glucose levels at any period after dextrin gavage(5). As a result, blood glucose decline at any period after 90 minutes following dextrin gavage could not readily be related to a possible insulin-like effect of the test material. However, insulin activity could be readily differentiated from spontaneous variation by employing the initial 90 minute, or ascending, portion of the blood glucose curve. The first blood glucose was usually obtained 4 ± 2 minutes after simultaneous dextrin gavage and intraperitoneal injection of test material. Succeeding blood glucose levels at 30 ± 10 and 65 ± 15 minute intervals were determined and the increments over the initial value calculated. Sufficiently diminished increments indicated insulin-like activity. However, a *precise* quantitative assay has not yet been achieved.

Experiments with fresh crystalline zinc insulin (Insulin Lilly, Iletin, U. 40), diluted in normal saline, indicated that 0.1 milli-unit showed no significant effect as compared with saline whereas 1-2 milli-units demonstrated an equivocal suppression of hyperglycemia. 4-5 milli-units showed a very significant effect, to the extent of nearly obliterating the rise in the blood glucose curve (Tables I and II).

Human plasma was prepared from 4 healthy young males who had been given 100 g glucose orally and bled 1½ hours later. The heparinized blood was spun in a refrigerated centrifuge and fresh samples were tested. Some plasma was also immediately frozen for later use. Tables I and II indicate that these plasma samples, in 0.25-1.0 ml doses, demonstrated no insulin-like activity as compared with normal saline.

Human plasma fractions tested were: a) Stable Plasma Protein Solution (SPPS), containing principally albumin and other proteins forming soluble zinc complexes; b) Protein Globulin Precipitate (PGP), a globulin-rich fraction precipitated by zinc and c) a gamma-globulin fraction extracted from PGP(6).§ The concentrations of globulin (PGP) and albumin (SPPS) fractions were 5:1 compared with the original plasma. About 4 of the gamma globulin samples represented a concentration 10-15 times that of the original plasma. Despite variation of response occurring with differing lots of mice and of plasma fractions, the data accumulated were sufficiently consistent to permit accurate statistical evaluation. Tables I and II demonstrate that the *only* statistically valid (P = <0.01) insulin-like activity, based on normal saline as the control material, was found in the globulin (PGP) fraction. The 65-minute, but not the 30-minute, increments of blood glucose reveal

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TABLE II. Insulin-Like Activity of Various Substances Measured by Comparison of Blood Glucose Increments during 65 Minutes.

Material tested	No. of determinations	Mean rise in blood glucose (mg %)	Significance of difference between means by "t" test — P value — Saline	Plasma
Saline	27	353	—	—
Insulin, 0.1 mU	4	262	>.3	—
" 1-2 mU	4	146	>.02; <.05	—
" 4-5 mU	5	29	<.001	—
Plasma	24	316	>.4	—
Gamma-globulin	7	285	>.4	>.6
Albumin (SPPS)	11	192	>.01; <.02	>.02; <.05
Globulin (PGP)	45	163	<.001	<.001

a similar clear-cut effect of globulin (PGP) as compared with whole plasma.

Effectiveness of the globulin (PGP) fraction did not appear dependent on maintenance of any particular dosage level so long as a minimum of 0.25 ml was administered. 0.3-0.6 ml was the usual dosage administered.

Discussion. With blood glucose increments measured at the 30-minute mark, there is less separation of globulin (PGP) from whole plasma and saline. This is probably a reflection of greater variation of glucose absorption and metabolism during the 30-minute period immediately following gavage. Thus, a more sensitive assay is achieved when blood glucose increments at 65 minutes are compared.

The mechanism of this insulin-like activity by globulin (PGP) may be related directly to the 5-fold concentration of the plasma fractions achieved by the fractionation procedure. However, the observation that the albumin (SPPS) and gamma-globulin samples, despite being concentrated to an equivalent or greater extent than the globulin (PGP), exhibit less insulin-like activity than globulin (PGP) suggests that it would be of value to investigate further the possible presence of more specific insulin-like factors in highly purified plasma fractions.

Summary. Modifications of the method of Anderson and associates for insulin bioassay are described. Plasma fractions, particularly globulin, demonstrate insulin-like activity by this technic.

We take this opportunity to thank Mr. Leston Nay for performing the hypophysectomies and Mrs. Nancy Meegan for tending the mice.

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Acetylation of P-Aminobenzoic Acid in X-Irradiated Rats. (21142)

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That the inactivation of Coenzyme A (CoA) may be of importance in the development of cellular damage in animals exposed to ionizing radiation has been suggested by several authors(1-3). It has been shown that: a) the protective effect of cysteamine is enhanced by simultaneous administration of pantothenic acid prior to irradiation(1); b) CoA preparations are superior to cysteamine in the pre-irradiation treatment of mice(4); and c) CoA is highly sensitive to oxidation by X-radiation *in vitro*(5). In regard to the first of these, small numbers of animals were used, and the results are far from conclusive. Further, the demonstration by Pierpoint and Hughes(6) that cysteamine is not involved in the biosynthesis of CoA in bacteria, suggests that any beneficial effects of pantothenic acid in supplementary radiation prophylaxis may be attributable to some other effect. The increased protection observed in mice injected with CoA prior to irradiation may be a reflection of the pronounced pharmacologic effects of the impurities in the CoA preparations employed(4). Finally, it has been shown several times that high radiation sensitivity of a compound *in vitro* does not necessarily bespeak a high sensitivity in the intact animal.

We have studied the capacity of the irradiated rat to acetylate p-aminobenzoic acid (PABA) and sulfanilamide, and have concluded that irradiation does not interfere with this process.

Methods. Female Sprague-Dawley rats, weighing 160 to 200 g, were used in these experiments. Because of differences in rates of excretion of the free and acetylated drugs, the renal vessels and ureters were ligated prior to intravenous injection of 50 mg/kg PABA or sulfanilamide. Blood samples (0.2

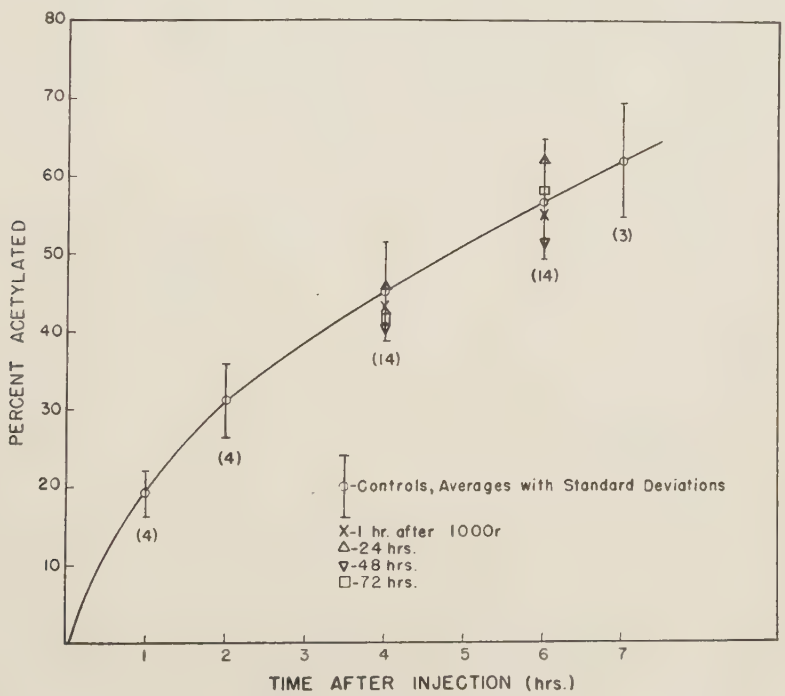

FIG. 1. Rate of acetylation of PABA in control and X-irradiated rats. Numbers of control rats indicated in parentheses.

ml) were taken at varying intervals after ligation and injection from irradiated and non-irradiated animals and were analyzed for free and total compound by the method of Bratton and Marshall(7).

Irradiated rats received 1000 r of X-irradiation at a rate of 225 r/min from a machine operated at 250 kv, 15 ma, 0.5 mm Cu and 3.0 mm bakelite filters, 27 cm distance. At 1, 24, 48, and 72 hours after exposure, groups of 4 rats were treated as above.

Results. Fig. 1 shows the percent acetylation of PABA at various times after injection. It is apparent that during the first 3 days after exposure the irradiated animals did not differ significantly from the controls in their ability to acetylate PABA, an indication that this particular reaction, requiring CoA, was not affected by irradiation. Similar results were obtained with sulfanilamide on a small number of animals.

Discussion. Acetylation of PABA and its analogues is considered to be a function of the liver, an organ generally believed to be radio-resistant. The fate of CoA in the sensitive hematopoietic tissues, the gonads, and the intestinal mucosa is not known; however, it appears that CoA, measured in terms of an *in vivo* function, is not indiscriminately destroyed. Van Heyningen *et al.*(8) have measured CoA levels in the lens after irradiation, and have found that the concentration decreased as opacities developed; however, the decrease is a delayed effect, and not an immediate action of X-radiation.

Summary. Rats exposed to 1000 r X-radiation retained their capacity to acetylate p-aminobenzoic acid and sulfanilamide for at least 3 days after exposure, an indication that Coenzyme A is not indiscriminately destroyed by whole-body irradiation.

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Intravenous Infectivity of Type 2 Poliomyelitis Virus in Mice.* (21143)

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After Armstrong(1) had demonstrated the susceptibility of mice to intracerebral infection with Type 2 poliomyelitis virus (Lansing strain), Casals, Olitzky and Anslow(2) were successful in adapting the MEF-1 strain of the same type intracerebrally in suckling mice. It was noted that the concentration of virus in the CNS increased with progressive serial passages in suckling mice. Similar observations have been reported by others(3,4) working with the MEF-1 strain of poliomyelitis virus in young hamsters as well as in young mice. Using the suckling mouse adapted line of the MEF-1 strain, Hammon, Cheever and Sather(5) have demonstrated the susceptibility of 4 to 15 days old mice to intraperitoneal infection. Similar results were observed by Moyer, Accorti, and Cox who inoculated their strain adapted to suckling hamsters intramuscularly or intraperitoneally into 21 to 28 day old mice(3). Though the precise portal of entry of poliomyelitis virus in

* Aided by a grant from The National Foundation for Infantile Paralysis.

man is still uncertain, it is most unlikely that it invades the CNS directly. However, experimental work with small animals has been limited to direct intraneural injection, for the most part, because of lesser susceptibility when the virus is given by other than neural routes. In experimental work that aims to reproduce natural condition of infection as closely as possible, a poliomyelitis strain inducing measurable response in small animals following peripheral infection, might be of considerable value. This report contains the details of a technic that has produced a line of the MEF-1 strain pathogenic for 4 to 6 weeks old mice when administered via the intravenous route; there is also described some of the characteristics of this virus.

Materials and methods. Criss-Cross Passages. The method applied to increase the infectivity of the MEF-1 strain when given intravenously consisted of serial criss-cross passages using tissue culture fluids for the intravenous inoculation in mice and passing the virus recovered from the cord of paralyzed mice into tissue cultures as described earlier (6). *Virus.* The experiment was started with cord material from one mouse that was found paralyzed in a group of 20 mice, 8 days after intraperitoneal inoculation with MEF-1 virus, which had undergone 6 tissue culture passages in this laboratory. The MEF-1 strain was identified as Type 2 poliomyelitis virus; (a) in tissue cultures, using immune sera representing each of the 3 types of poliomyelitis virus; (b) mice immunized with living or with inactivated Type 2 virus were resistant to intravenous challenge with this line. *Animals.* CFW mice of the same source with an average age of 4 to 6 weeks were used for intravenous, intraperitoneal and intracerebral inoculations. The inoculum consisted of tissue culture fluid in a volume of 0.25 ml for the peripheral routes and 0.025 ml for the intracerebral route unless otherwise stated. The response (paralysis or death) as well as the site of paralysis was recorded daily. *Tissue Cultures.* The technic employed for the preparation of tissue cultures was essentially the same as described by Dulbecco and Vogt(7). The cultures were incubated stationary in small bottles at 36°C and inoculated when a complete tissue sheet covered the bottom of the culture vessels. The fluids were harvested as soon as all tissue was degenerated which usually occurred after 3 to 4 days at 32° or 36°C. Tissue culture fluid from the 4th criss-cross passage induced paralysis in 80 to 100% of mice within 10 days when 0.25 ml was injected into a tailvein. A group of 40 mice infected in this way were used to study the distribution of the inoculated virus in serum, brain, cord and feces. Material from 2 mice was pooled for each determination made at 24 hour intervals after infection. Histological examinations were done on cord and brain specimens. The *serum* was obtained from mice by amputation of right fore-leg. The blood was collected in isotonic saline giving a final serum dilution of approximately 10^{-1}. The blood cells were removed by centrifugation and the supernatant fluid containing the serum was titrated for antibody in tissue cultures. *Brains and spinal cords* were removed aseptically and a 1% tissue suspension in distilled water prepared, centrifuged at 2000 RPM for 30 minutes and the supernate considered a 10^{-2} dilution of the original material. *Feces* were ground in a mortar. A 10% suspension by weight was made up in buffered saline (pH 7.2). The large particles were removed by centrifugation at 2000 RPM for 15 minutes. The supernatant fluid was separated and streptomycin and penicillin were added giving a final concentration per milliliter of 500 units and 0.1 mg, respectively. This material was titrated in 10-fold dilution steps in tissue cultures.

Results. Fig. 1 contains the response-time distribution data for mice infected intravenously with undiluted, and with a 10-fold dilution of tissue culture fluid from the 6th criss-cross passage. Forty mice were used for

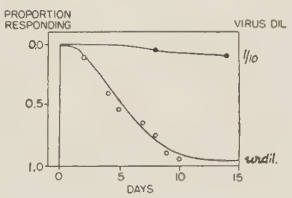

FIG. 1. Response time distribution of mice infected with MEFl intravenously.

TABLE I. Proportion of Mice Responding during Observation Period of 2 Weeks, Inoculated by Different Routes with Different Pools and Doses as Compared to Proportion of Tissue Cultures Responding to Inoculation of Various Virus Dilutions. 50% endpoints are estimated graphically from plots on log probability paper.

Route and dose (ml)	No. criss-cross passages	Negative logarithms of the virus dilutions inoculated 0	1	2	3	4	5	6	7	8	ID_{50}/ml	Geometric mean of ID_{50}/ml values
i.c., .025	6	—	10/10	10/10	9/9	4/10	—	—	—	—	$10^{5.4}$	$10^{4.9}$
	8	—	10/10	10/10	5/10	0/10	—	—	—	—	$10^{4.4}$	
i.v., .25	6	10/10	2/10	0/10	—	—	—	—	—	—	$10^{.9}$	$10^{1.0}$
	8	13/19	6/20	0/20	—	—	—	—	—	—	$10^{1.1}$	
i.p., .25	6	3/10	0/10	—	—	—	—	—	—	—	$10^{.1}$	$10^{.3}$
	8	4/10	1/10	—	—	—	—	—	—	—	$10^{.4}$	
Tissue cultures, .5	0	—	—	—	—	6/6	6/6	5/6	2/6	0/6	$10^{7.0}$	$10^{7.9}$
	6	—	—	—	—	6/6	6/6	6/6	6/6	0/6	$10^{7.8}$	
	8	—	—	—	—	6/6	6/6	6/6	6/6	1/6	$10^{7.9}$	

each dilution and 0.5 ml injected. The 50% response-time end-point of paralysis or death, estimated from the figure, is approximately 5 days. The distribution of the site of paralysis is as follows: 24% showed flaccid paralysis beginning in one or both hind-limbs, 43% in one or both fore-limbs, 25% were found dead or paralysis was so far advanced that the beginning site could not be determined, and 6% remained without any abnormal signs during an observation period of 15 days.

The proportion of survivors in the titration of tissue culture material from the 6th and 8th criss-cross passage in mice inoculated by different routes as well as titrations in tissue cultures are recorded in Table I. The ID_{50} values can be used to estimate roughly the ratio of equally effective doses, *i.e.*, the potency by the different routes relative to the tissue culture method. The relationship may be represented as follows:

ID_{50} in tissue cultures:	Intracer.	Intrav.	Intraper. route
1	10^3	10^7	$10^{7.4}$

Fig. 2 shows the distribution of virus in serum, brain, cord and feces at various intervals after intravenous inoculation. It can be seen that the virus disappeared rapidly from the serum within the first 24 hours. While the virus content of the cord increased after a 24 hour lag period, an even more prolonged lag period was observed when brain material was titrated. No virus was recovered from the feces during a 48 hour period following infection but material collected between 48 hours and 72 hours showed the presence of low concentrations of virus.

On histological examinations† of cord and brain from animals paralyzed at various intervals after infection no histological lesions could be detected in cortical areas of brain specimens collected up to 7 days after infection. But typical lesions were observed in the anterior horns of the spinal cords of animals found paralyzed on the 5th day or later.

Discussion. The results presented demonstrate the susceptibility of 4 to 6 weeks old CFW mice to peripheral infection with a line of the MEF-1 strain of Type 2 poliomyelitis

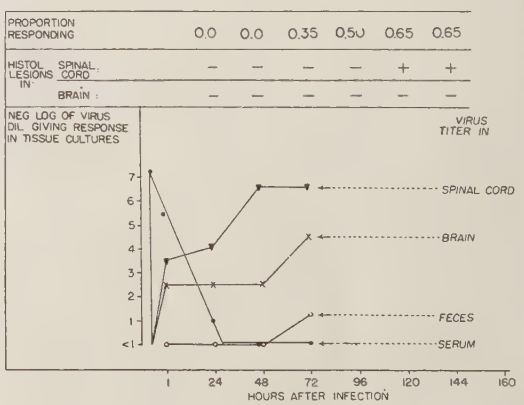

FIG. 2. Distribution of virus in spinal cord-brain-serum and feces of non-paralyzed mice at various intervals after intravenous infection.

† Dr. L. J. Lewis' help in the examination of the histological preparations is gratefully acknowledged.

virus. In order to produce this effect in mice, virus of relatively high infectivity was used. Following criss-cross passages between tissue cultures and mice the infectivity of this strain was increased almost 10-fold when titrated in tissue cultures.

The dissociation between ID_{50} inducing paralysis or death when given peripherally or centrally is striking. From this it might seem that only small amounts of virus injected intravenously will reach the CNS. Poliomyelitis virus has been demonstrated in other than nervous tissues following infection in man as well as in animals (blood, lymphnodes and spleen). Though it is questionable whether poliomyelitis virus multiplies in non-neural tissues *in vivo* the presence of virus in these organs following intravenous inoculation may result in a rapidly developing immunity which might interfere with the occurrence of the paralytic disease when virus doses are given that result in an incubation period of more than 10 days.

Summary. Poliomyelitis virus Type 2 has been successfully propagated in mice following intravenous inoculation. 10^7 ID_{50} tissue culture doses were required to produce 50% response (paralysis or death) after intravenous inoculation in mice. The intravenously inoculated virus disappeared from the serum after 24 hours but was recoverable from spinal cord, brain and feces.

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Influence of Bean Trypsin Inhibitors on Thromboplastic Properties of Trypsin and Hypochlorite Treated Trypsin.* (21144)

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That trypsin is capable of accelerating the coagulation of blood is well known(1,2) and it has been repeatedly observed that bean trypsin inhibitors retard coagulation(3-6). The present report deals mainly with the thromboplastic properties of trypsin or hypochloric treated trypsin combined with bean trypsin inhibitors. The differentiation of the inhibitors used is briefly considered.

Methods. Coagulation time was determined as described earlier(7). Proteolytic activity was estimated according to the method of Kunitz(8). The inhibition of proteolytic activity was measured by determining the amount of inhibited enzyme required to accomplish the same degree of digestion of a given solution of casein as did a known amount of the untreated enzyme. Inhibitor solutions were added to enzyme solutions at pH 7.4 ten minutes before use. The globulin soy bean trypsin inhibitor, GSTI, was prepared according to the method of Kunitz(9) who has described the properties of the crystalline material(8). The navy bean trypsin inhibitor, NTI, was prepared as follows: 800 g of finely ground navy beans were extracted with 4 liters of water at 50°C for 18 hours. After centrifuging, trichloroacetic acid was added to the supernate to 2.5% concentration. The preparation was heated to 80 to 85°C for 5 minutes and centrifuged while hot. The

* These studies were aided by a contract with the Office of Naval Research and by the Alexander and Margaret Stewart Trust.

supernate was adjusted to pH 6.5 with NaOH after cooling to 25°C. Sufficient 95% ethyl alcohol was added to bring the concentration to 60%; the resulting precipitate was discarded after centrifuging, and the alcohol content was increased to 75%. After standing overnight at room temperature most of the supernatant solution was removed by siphoning; the precipitate was then collected by centrifuging and dried in a vacuum desiccator. Yields varied from 0.3 to 0.5% of the bean meal. Preparations of greatest activity were obtained from newly harvested beans. The protease soy bean trypsin inhibitor can be prepared by the above procedure except for the adjustment to pH 9 prior to adding alcohol. Hypochlorite-treated trypsin was prepared as described earlier(7). Rabbits were used because of their marked susceptibility to pulmonary and cardiac embolism with the intravenous injection of trypsin. The term trypsin refers to crystalline trypsin unless otherwise indicated and Cl-trypsin refers to hypochlorite-treated trypsin. Throughout this investigation observations were made with both GSTI and NTI with comparable results. Data are given for the former because of its crystalline nature although the properties of the latter may offer advantages for *in vivo* administration.

Results. The intravenous administration of the bean trypsin inhibitors with trypsin causes considerably less fatal embolism in rabbits than does the enzyme alone. For example, the injection of 5 mg of trypsin/kg was fatal in all of 10 rabbits as was 10 mg/kg in an equal number. However, 10 daily injections of the enzyme in 10 mg/kg doses with an equal amount of GSTI was tolerated by 10 animals without response. The emboli observed in the fatal cases were large and unmistakable, frequently filling the pulmonary arteries and right auricle. They were observed immediately after cessation of respiration which occurred within a few minutes after injection. Death was preceded by marked symptoms of anoxemia.

TABLE I. Thromboplastic and Proteolytic Activities of Inhibitor-Enzyme Complexes *In Vitro*.

Enzyme: inhib. ratio	% of control coagulation time*		Proteolytic activity†	
	Trypsin + GSTI	Cl-trypsin + GSTI	Trypsin + GSTI	Cl-trypsin + GSTI
1:3	142	161	0	0
1:2	70	121	0	0
1:1	21	40	2	0
1:0.5	3	25	15	5

* In each case 0.6 mg protease used/ml of plasma.

† % of the uninhibited activity of the enzyme as estimated by casein digestion.

TABLE II. Thromboplastic Effect of Inhibitor-Enzyme Complexes Injected by Jugular Vein.

Enzyme, mg/kg	GSTI, mg/kg	% of control coagulation time after inj. 5 min.	60 min.	180 min.
		Trypsin		
2*	0	26	42	103
5	5	28	47	51
5	10	45	57	60
		Cl-trypsin		
5	0	40	47	71
5	5	89	76	48
5	10	100	102	109

* 5 mg/kg caused fatal embolism.

Although an equal weight of GSTI administered with trypsin reduced embolism the complex retains marked clot accelerating properties. This was observed *in vitro* (Table I) and after intravenous administration (Table II). Appreciable clot acceleration occurs even when the enzyme:inhibitor complex shows little or no proteolytic activity; and the injection of the uninhibited enzyme in amounts sufficient to provide proteolytic capacity equivalent to that of the 1:1 complex did not alter clotting time. On the other hand, relative protection from embolism is observed even with an enzyme:inhibitor complex showing considerable proteolytic activity. Commercial trypsin differs from the crystalline enzyme in that approximately half of its original proteolytic activity is retained in the presence of 2 or 2.5 times as much GSTI or NTI. Yet the inhibitors decidedly diminish embolism (Table III) in spite of this residual proteolytic activity. Again, markedly decreased clotting times were observed.

Therefore, it would appear that the enzyme:inhibitor complex does not influence the blood clotting mechanism through its initial

TABLE III. Influence of Bean Trypsin Inhibitors on Trypsin Embolism in Rabbits.

No. of animals	Material inj. by ear vein	No. of fatalities	No. with gross emboli	Amt of trypsin inj., mg/kg*		
				Avg total	Max	Min
44	Trypsin (commercial)	40	38	8	19	2
20	" GSTI (1:2)	2	1	20	30	10
20	" NTI (1:2.5)	3	1	22	27	8
20	Cl-commercial trypsin	14	14	18	31.5	3
23	*Idem*, NTI (1:2.5)	5	5	14.5	22	3

* Injected at rate of 1 mg enzyme/kg/15 min.

free proteolytic effect; although plasma activated with acetone(7) did show a significant increase in proteolytic activity after the complex was added *in vitro* or injected. This occurred without change in the natural inhibitor level of plasma prior to acetone treatment. Continued study of the problem may provide further information relative to the controversy over the participation of proteolytic factors in the natural blood clotting process.

The thromboplastic properties of the enzyme:inhibitor complex in which hypochlorite-treated trypsin is substituted for the untreated enzyme are similar to those described above (Tables I, II, III). The properties of uninhibited Cl-trypsin have been described(7). Although the bean trypsin inhibitors do not diminish the hypotensive effect of trypsin(10) such diminution in marked degree results from treating the enzyme with hypochlorite(7). Therefore, suitable amounts of the crystalline enzyme so treated and combined with the GSTI has low hypotensive and proteolytic activities with an appreciable thromboplastic effect. Optimal quantities for the thromboplastic effect of the 1:1 complex *in vitro* are given in Table IV. The duration of decreased coagulation time after injecting trypsin or Cl-trypsin with an equal amount of inhibitor is generally more prolonged than with the uninhibited enzyme administered in sufficient amounts to produce about the same initial clot acceleration.

TABLE IV. Thromboplastic Activity of Varying Amounts of Cl-crystalline Trypsin in Presence of Equal Amounts of GSTI and without Inhibitor *In Vitro*.

mg enzyme/ ml plasma	% of control coagulation time	
	With GSTI	Uninhibited
.015	51	8
.044	48	7
.291	43	6
.582	45	6
1.165	75	4
1.75	78	5
2.33	93	5
2.91	105	Incoagulable

In view of the differing behavior and properties of the GSTI and the proteose inhibitors of soy and navy beans, and since they are commonly confused, further brief comment on their differentiation is given. In the course of investigation of the remarkable resistance of navy bean starch to enzymatic digestion(11) enzyme inhibitors were detected in small amounts while working with the fatty fractions of both navy and soy beans(12). It was found that the inhibitors are much more abundant in aqueous extracts and that, although part of the trypsin inhibitor fraction of soy beans(13) is soluble in 60% alcohol, soy beans also contain an alcohol insoluble fraction(14). The globulin soy bean fraction later crystallized by Kunitz(15) corresponds with the latter(16) and the former is probably the fraction of Ham and Sandstedt(17) which is soluble in alcohol and lost on dialysis. The NTI(13,16) is soluble in 60% alcohol and is not crystallized by the method applicable to the GSTI. The NTI has the properties of a proteose: it precipitates in cold 0.25% nitric acid, redissolves with heat and reprecipitates on cooling, is soluble in 60% alcohol, precipitates in 0.5 saturated $(NH_4)_2SO_4$, and is more soluble in hot trichloroacetic acid than in cold. It dissolves in buffer solutions more readily than does the GSTI. The present NTI preparations give positive biuret, ninhydrin and Millon tests. The proteose soy bean trypsin inhibitor has similar properties; however, present preparations give a negative Millon's test and since it inhibits trypsin much less actively than do the GSTI or NTI it has been

used in this laboratory less frequently. The NTI and proteose soy bean trypsin inhibitor both inhibit chymotrypsin much more actively than does the GSTI.

Summary. Soy and navy bean trypsin inhibitors considerably reduce the incidence of fatal trypsin embolism in rabbits with the retention of marked thromboplastic activity by the inhibitor-enzyme complex. The thromboplastic effect is observed with little or no proteolytic activity and the relative protection against embolism is apparent even with extensive proteolytic activity. Similar observations were made with the bean trypsin inhibitors and hypochlorite-treated trypsin, the latter having been shown earlier to have considerably less hypotensive effect than untreated trypsin. The differentiation of the soy and navy bean trypsin inhibitors is briefly considered and an improved method of preparing the navy bean inhibitor is given.

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Distribution and Excretion of Radioactivity after Administration of Morphine-N-methyl C^{14} to Rats.* (21145)

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Our knowledge of the tissue distribution and excretion of morphine is limited because available analytical methods are not sensitive enough to determine tissue levels after the administration of a single therapeutic dose. The synthesis of morphine-N-methyl-C^{14}(1) has made it possible to follow the drug through the body using tracer technics. This report deals with tissue distribution and excretion of carbon-14 in rats after administration of minimal analgetic doses, as well as an investigation of N-demethylation of morphine *in vitro*.

Methods. In vivo studies: Adult Wistar-type rats of both sexes weighing approximately 200 g were given 5 mg/kg of suitably diluted morphine-N-methyl-C^{14}HCl subcutaneously. This constituted a minimal analgetic dose in the animals studied. The carbon-14 in the tissues, excreta and expired air was determined as previously described(2). For biliary tract excretion studies a rat was anesthetized with ether and a polyethylene catheter was inserted into the common bile duct through an uppermidline abdominal incision. The abdomen was closed, the animal was placed in a restraining cage and labeled morphine was administered after the animal had fully recovered from the anesthetic and bile was flowing freely.

Six female rats were given a total of 45 mg cyclopentyl testosterone propionate in three 15 mg subcutaneous doses over a 38-day period prior to studies on the effect of androgens. Two rats were made tolerant to morphine by daily injections for 7 weeks beginning

* Aided by a grant from the National Institutes of Health, Bethesda, Md.

TABLE I. Percent Radioactivity Recovered from Tissues, Excreta and Expired Air after Injection of 5 mg/kg Morphine-N-methyl-C^{14} Subcutaneously.

Time of sacrifice Sex	1 hr ♂	6 hr ♀	24 hr ♀	48 hr ♂
				(1st 7 hr)
Expired air	1.1	.2	.6	8.2
Liver	2.3	.4	.3	
Stomach	6.0	.1	.04	
Small intestine	15.7	3.4	6.5	.3
Large "	.5	19.6	16.5	.2
Feces			24.0	26.7
Kidneys and bladder	1.3	.8	.2	
Urine	17.8	63.6	52.1	Lost
Left leg	9.8	1.1	.2	
Other	39.0	5.4	1.2	<.5
Total	93.5	94.6	101.6	

with a dose of 10 mg/kg and working up to 40 mg/kg after 5 weeks. *In vitro* studies: Liver slices approximately 0.5 mm thick were cut freehand and 800-1000 mg were suspended in 10 ml of extracellular Krebs-Ringer solution in modified Warburg flasks at 37.2°C. Oxygen or other gas as specified was passed through the flasks into NaOH towers for absorption of carbon dioxide. At the end of the experiment carbon dioxide in solution was liberated by acidification of the Ringer's solution. Labeled morphine was added to make a final concentration of 10 μg per gram of liver, a value derived from the *in vivo* studies.

Results. 1) Distribution of radioactivity after subcutaneous injection: These results have been reported in part(3) and are summarized in Table I. Up to 20 individual tissues were analyzed from some animals but for simplicity of presentation only those containing significant amounts of radioactivity are tabulated. Absorption was not quite complete at one hour; the left hind leg (site of injection) contained 9.8% of the dose compared to 2.5% in the opposite leg. Brain and spinal cord at one hour contained only traces of activity by the method used; other tissues not directly concerned with excretion showed low levels of activity. Concentration occurred chiefly in organs of excretion as evidenced by the large amounts of activity present in the urine and gastrointestinal tract one hour after drug administration. By 6 hours almost two-thirds of the activity was excreted in the urine and by 24 hours 99% was in the urine, feces or gastrointestinal tract. The expired air of the males in this series contained appreciable amounts of activity indicating an N-demethylating or transmethylating process while females excreted minimal amounts by this route.

2) Biliary excretion: 62.6% of the injected carbon-14 was recovered from bile collected from a female rat during the 6-hour period following injection of 5 mg/kg of labeled morphine. Urine collected during the same period contained 18.1% of the dose. These figures are practically the reverse of those obtained in the intact animal and suggest an entero-hepatic circulation of morphine or its metabolites following the initial excretion by the liver in the bile. No appreciable amount of activity was present in the gastrointestinal tract of this animal. The material excreted in the bile consisted of both free and bound morphine. Colorimetric determinations(4) before and after acid hydrolysis under 15 lb pressure showed that the major portion of the morphine recovered in the bile is present in the bound form. Similar results have been reported for dogs(5) but in man all the morphine in the bile was conjugated(6).

3) Pulmonary excretion of $C^{14}O_2$ by intact, androgen-treated and morphine-tolerant rats is shown in Table II and Fig. 1. The expired air from untreated males and females, from a male and females treated with cyclopentyltestosterone propionate and from a tolerant male rat was collected for 6 hours after subcutaneous administration of 5 mg/kg labeled morphine and its radioactivity measured. A 1-hour collection was made on another tolerant male rat. The amount of $C^{14}O_2$ liberated by the treated females was similar to that excreted by the males while the amount from untreated females was minimal, and that from the treated male quite high, confirming the data obtained previously(3). Pulmonary excretion by morphine-tolerant rats 1 and 6 hours after injection was similar to that of normal rats.

4) *In vitro* studies: The liberation of $C^{14}O_2$ from rat liver slices treated with morphine-N-methyl-C^{14} under various conditions is shown in Table III. It is apparent that the sex difference observed *in vivo* is reproduced *in*

TABLE II. Percent Radioactivity Recovered from Expired Air during 6 Hr after Injection of 5 mg/kg Radioactive Morphine Subcutaneously.

Untreated rats		Cyclopentyl testosterone treated rats		Morphine-tolerant rats
Males (5)	Females (8)	Males (1)	Females (4)	Males (2)
4.77	.50	11.4	4.1	7.4-6 hr .7-1 hr

vitro. Furthermore, the amount of $C^{14}O_2$ liberated by liver slices approximates that recovered from expired air *in vivo.* Added testosterone failed to increase $C^{14}O_2$ liberation by liver slices from female rats and depressed it in the liver slices from male rats. The latter effect is probably related to the depression of tissue respiration produced by testosterone(7). Methionine in the amount used did not alter the rate of "demethylation", nor was it appreciably depressed by an amount of ethionine sufficient to make a 20:1 ethionine-methionine ratio based on the amount of methionine in liver(8). Dibucaine in the concentration used produced only inhibition of "demethylation". This amine oxidase(9) and cytochrome(10) inhibitor was included in this series because independent experiments showed that it produced a diphasic effect on the QO_2 of liver slices from fed rats, first stimulating and then inhibiting oxygen uptake. No parallel effect was observed on $C^{14}O_2$ liberation. Finally, the rate of "demethylation" was not affected by N-allylnormorphine but was completely inhibited by anaerobic conditions or homogenization in a Potter homogenizer.

Discussion. The general pattern of morphine excretion in the rat was followed by tracer methods. The presence of $C^{14}O_2$ in the expired air shows that morphine is in part "demethylated" in the male rat. This was confirmed in man(11) although no sex differences were found. Thus, some morphine is probably converted to normorphine which may be excreted either free or conjugated. This process resembles that found with meperidine (12) and probably with codeine(13), but is probably relatively unimportant insofar as the detoxification of morphine is concerned. This belief is strengthened by the finding that tolerant animals do not show increased "demethylation" and that morphine has been reported to be more toxic to mature male rats than to mature females(14). It is of interest that N-allylnormorphine which is conjugated by the liver at the same rate as morphine, and acts as a competitive inhibitor(15), does not seem to be effective in blocking the "demethylation" of morphine.

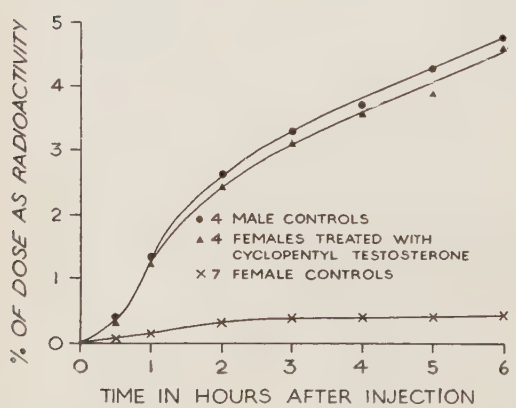

FIG. 1. Radioactive CO_2 excreted by pulmonary route after subcutaneous administration of 5 mg/kg labeled morphine.

It is most interesting to find a process in the liver which is stimulated by androgens. Very little is known regarding the influence of castration or androgen administration on liver enzyme content(16). Data of this type might help in determining the systems involved in "demethylating" morphine. Other compounds such as the xanthines, cocaine, mesantoin, mephobarbital, aminopyrine, and ephedrine have been shown to be demethylated in the body but no sex differences were reported.

Pulmonary excretion of carbon-14 by tolerant animals is not markedly different from that by normal animals as was also found in man(11). In the rat and dog, previous work has shown that an important fraction of the administered morphine is not recoverable from the excreta of tolerant animals(5,17). By using labeled morphine and comparing radioactive and colorimetric values it should be possible to locate this "missing" fraction so as better to understand the changes induced by

TABLE III. Percent Radioactivity Liberated in One Hour as $C^{14}O_2$ by Rat Liver Slices *In Vitro*.

Animals	Substances in flask	%
Males, untreated (7)	10 γ M*	Avg 14.8 (range 12.0 -22.0)
Females, " (3)	10 γ M	" .29(" .17- .52)
" , treated with testosterone, cyclopentyl propionate (2)	10 γ M	21.0, 23.7
Male, untreated	10 γ M 10 mg testosterone propionate suspension	3.6
Female, "	10 γ M 1 mg testosterone propionate suspension	.33
Male, "	100 γ M	13.6
	10 γ M 10 mg methionine	12.0, 12.7
	10 γ M 10 mg ethionine	10.5
	10 γ M—control	12.1
	Atmosphere of nitrogen 10 γ M	0
	10 γ M .002 M dibucaine	4.2, 3.1
	10 γ M 20 γ N-allyl normorphine	12.6
	10 γ M Liver ground to a brei	0

* M = Morphine-N-methyl-C^{14}.

the development of tolerance.

Summary. After the administration of morphine-N-methyl-C^{14} to rats, radioactivity concentrated chiefly in the urinary and intestinal tracts with only minimal amounts in the central nervous system. Urinary excretion predominated but there is evidence for an entero-hepatic circulation of morphine or its metabolites following initial excretion by the liver in the bile. Male rats excreted significantly more radioactivity via the pulmonary route than did females. Treatment of females with cyclopentyl testosterone increased their pulmonary excretion to the male level. Liberation of $C^{14}O_2$ by liver slices from male and female rats to which morphine-N-methyl-C^{14} was added *in vitro* followed the same pattern.

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Effect of Acetazoleamide (Diamox) on Glucose Tolerance. (21146)

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Acetazoleamide (Diamox),* a carbonic anhydrase inhibitor, is now often employed for the diuretic effect on the kidney which results through enzymatic blocking produced by this compound(1). The widespread participation of carbonic anhydrase in the enzymatic functions of tissue other than the kidney, namely, erythrocytes(2-4), stomach(5-7), pancreas (8), and brain(9), has centered considerable attention upon these organs in the clinical uses of acetazoleamide.

This investigation was undertaken following the observation of insulin shock in a patient with diabetic nephropathy, who had been given acetazoleamide as a diuretic. Previously she had been easily and well controlled with 25 units of NPH (Lilly) insulin. She was eating an adequate daily diet consisting of 1800 calories composed of 180 g of carbohydrate, 90 g of protein, and 80 g of fat. It was noted on 3 separate occasions that insulin shock occurred approximately 3 to 5 hours following the administration of 0.5 g of acetazoleamide. The reaction was promptly corrected by intravenous infusion of 50 cc of 25% glucose solution. To determine the cause for this reaction this preliminary study was undertaken in an effort to evaluate the effects, if any, of acetazoleamide on glucose tolerance of normal subjects.

Method. The first subjects studied were 6 normal, healthy medical students on whom 2 oral glucose tolerance tests were performed by the method to be described later. The first test was used as a control; 0.5 g of acetazoleamide was administered orally 30 minutes before the second test. There was no apparent difference in the results of these 2 tests except an indication that acetazoleamide might cause a lowering of blood sugar levels after 2 to 3 hours; therefore, in later tests acetazoleamide was administered 2 to 3 hours before glucose was administered. These 6 subjects are not included in any of the figures and tables.

For this study 2 glucose tolerance tests were performed on 14 non-diabetic medical and psychiatric patients, the first test being used as a control and the second followed 2½ to 3½ hours after one oral dose of acetazoleamide. Nine patients received the standard oral glucose tolerance test using 100 g of glucose after a minimum fasting period of 12 hours. Blood samples were collected at fasting, 30-, 60-, 120-, and 180-minute intervals (Fig. 1). Five patients received the intravenous glucose tolerance test after a minimum fasting period of 12 hours. Glucose, 0.5 g per kg of body weight, was administered as a 20% solution, and blood samples were collected at fasting, 30-, 60-, 90-, and 120-minute intervals (Fig. 2), following the intravenous infusion of the glucose solution. In 3 to 5 days the same procedure was repeated on all patients with one oral dose of acetazoleamide 2½ to 3½ hours before administration of glucose. Acetazoleamide was administered in doses of 0.5 and 1.0 g with no difference in the glucose tolerance curve.

All of these patients had been maintained on a regular hospital diet, containing a minimum daily average of 300 g of carbohydrate, for 3 to 5 days prior to performing the first test.

The concentration of blood glucose was determined by a modified Folin-Wu method using the spectrophotometer(10). Urinary sodium and potassium excretions were determined on 5 subjects during both the control test and following the acetazoleamide administration. Quantitative urinary sugar determinations after acetazoleamide administration failed to reveal an increase in glucose excretion over the control glucose tolerance test in any of the groups.

Discussion. In this investigation it was shown in 5 subjects that urinary potassium

* Furnished by Lederle Laboratories Division, American Cyanamid Co.

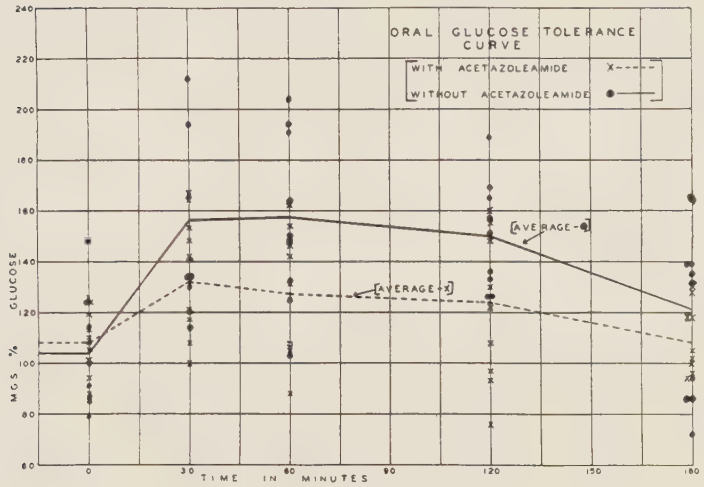

FIG. 1.

excretion was decreased after administration of acetazoleamide during glucose tolerance test (Table I); however, it has been reported that in normal subjects acetazoleamide increased urinary potassium excretion(11). Friedberg *et al.*(12) have clearly shown that in patients with congestive heart failure and receiving acetazoleamide, urinary potassium was increased by approximately 50%. An explanation for the diminished potassium in our investigation may be due to increased glycogenesis. The effect of insulin and glucose on urinary potassium is well known, however, the exact mechanism is poorly understood. It is thought that the migration of potassium ions from the intracellular spaces into the cells is related to glycogenesis. Therefore, a glycogenic effect initiated by acetazoleamide may be suggested by the decreased urinary potassium excretion.

The apparent difference between the oral and intravenous glucose curves (Fig. 1 and 2) following acetazoleamide administration suggests that gastrointestinal absorption of glucose may also be influenced by acetazoleamide. By what mechanism it would be operative is not known, unless some derangement in the rate of phosphorylation is produced.

It has also been demonstrated that sulfonamides, by competing with the thyroid for iodine, prevents the formation of diiodotyrosine, and consequently thyroxine(13). Whether this inhibition by acetazoleamide, (theoretically 50 to 400 times as potent a carbonic anhydrase inhibitor as sulfonamide) is of significant degree to influence carbohydrate metabolism is purely speculative.

Summary. Two glucose tolerance tests were performed on each of 14 subjects (9 oral glucose tolerance tests, 5 intravenous glucose

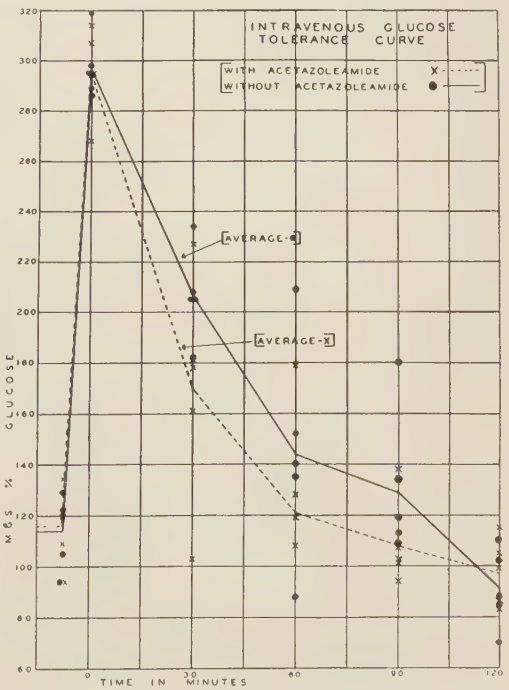

FIG. 2.

TABLE I. Urinary Sodium and Potassium Excretions. 5 subjects in each series.

Total urinary Na excretion		
Normal	Acetazoleamide	% increase
4.4 MEQ	58.4 MEQ	1225
10.5	76.2	635
49.0	82.3	68
15.5	40.2	58
54.6	121.7	123
	Avg % increase	440
Total urinary K excretion		
Normal	Acetazoleamide	% decrease
12.3 MEQ	4.9 MEQ	60
7.1	3.8	47
7.2	2.4	67
8.4	1.6	81
11.0	5.8	47
	Avg % decrease	60

tolerance tests). The first glucose tolerance test was used as a control. A second test was performed 2½ to 3½ hours after the administration of acetazoleamide. These tests showed a tendency for the acetazoleamide to increase the oral glucose tolerance. The administration of acetazoleamide 2½ to 3½ hours prior to the intravenous administration of glucose did not result in as marked a degree of change in the glucose tolerance curve. After administration of acetazoleamide to patients receiving oral glucose tolerance tests a diminution of the urinary potassium excretion was observed.

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Possible Occurrence of Multiple Antigens in Type 2 Poliomyelitis and GDVII Mouse Encephalomyelitis Viruses.* (21147)

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(Introduced by S. A. Koser.)

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The occurrence of at least two distinct antigens has been established for a number of mammalian viruses(1) and for *B. coli* T2 bacteriophage(2) by complement fixation and precipitin tests. The viruses for which such antigens have been separated and characterized are of relatively large size ranging from about 100 mμ to 450 mμ. However, very little information is available concerning the presence of multiple antigens in viruses considerably smaller in size. The foot and mouth disease virus, one of the smallest known viruses, has been found to have 2 complement fixing components(3,4); one associated with virus particles (infectivity) which measures about 20 mμ in diameter on the basis of sedimentation studies, and the other a smaller component of 6.5 mμ(4).

This report concerns the possible presence of 2 complement fixing antigens in GDVII mouse encephalomyelitis virus, estimated to be

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TABLE I. Comparative Complement Fixing Activity of Saline-Ether and Acetone-Ether Extracts of Infant Mouse CNS Infected with MEFl Virus.

Serum	Infant mouse passage	Antigen: Saline-ether Serum titer	Antigen: Saline-ether Antigen titer	Antigen: Acetone-ether Serum titer	Antigen: Acetone-ether Antigen titer
Type 2 anti-MEFl mouse pool 105105	67, 69	1:256 or >	1:2	1:512	1:8
Type 2 anti-Lansing monkey 35308	74	1:128	1:1	1:128	1:16
Type 2 anti-Lansing monkey 95305	76	1:4096	1:12	1:4096	1:48
Type 2 human	76	<1:8	0	1:128	1:12

Infectivity titer of saline-ether antigens for 3- to 4-wk-old mice following intracerebral inoculation ranged from $10^{-4.9}$ to $10^{-5.5}$ ID_{50} per .03 ml inoculum for 8 suspensions from 67th through 76th infant mouse passages. Infectivity titer for 4 suspensions of acetone-ether antigens varied from $10^{-1.8}$ to $10^{-3.7}$ ID_{50}.

7 to 12 mμ in size by ultrafiltration(5,6), and type 2 poliomyelitis virus, 12 to 23 mμ by ultrafiltration, and 23 to 30 mμ by sedimentation and electron microscopy(6,7). The occurrence of distinct antigens in these small viruses would be of fundamental significance. In addition, knowledge of the antigenic structure of poliomyelitis virus is of practical importance in view of the variation of complement fixing antibody in poliomyelitis patients (8) and application of the complement fixation reaction in epidemiological studies(9).

Materials and methods. Infant mouse brains and upper spinal cords infected with MEFl poliomyelitis and GDVII viruses served as the source of complement fixing (CF) antigens. The MEFl virus was kindly supplied by Dr. Casals in its 63rd infant mouse passage. Antigens were prepared from the 67th through 76th passage. GDVII virus, maintained in young adult mice, was highly virulent for infant mice. The incubation period varied from 2 to 7 days in the first 3 passages, but on subsequent passages it was, almost without exception, 2 to 3 days. Signs of disease were marked weakness, cyanosis, death, and occasionally paralysis. The *antigens* were prepared as follows: Following intracerebral inoculation of 2- to 3-day-old mice, the brains and cords were harvested from mice with paralysis 2 to 4 days after inoculation with MEFl virus, and marked weakness or paralysis after inoculation with GDVII virus. The infected CNS tissue was extracted with either saline-ether(10) or acetone-ether(11) and then used as antigen following centrifugation at an average relative centrifugal force of approximately 13,500 g for 30 minutes. Fifty to 100 brains and cords were used for the saline-ether and usually 200 to 400 for the acetone-ether extract antigens. *Type 2 poliomyelitis* sera were prepared by immunization of monkeys with monkey spinal cord infected with the Lansing strain and adult mice with the infant mouse adapted MEFl virus. Sera were also obtained from poliomyelitis patients. The GDVII sera were prepared by immunization of mice with adult mouse virus. All sera were inactivated at 60°C for 20 minutes for the CF tests. The *CF tests* were performed employing two 50% units of complement. Readings were made in a Coleman Junior Spectrophotometer at a wave length of 550 mμ. For titration of antigen, the antigen was diluted and the serum held constant. Serum titers were established by the reciprocal procedure. In addition, a large number of tests were carried out by using various dilutions of antigen against various dilutions of serum. The tests were set up in 0.3 ml volume (0.1 ml serum, complement and antigen), held at 5 to 10°C for 16 to 24 hours and followed by the addition of 0.2 ml sensitized cells, and incubation at 37°C for 30 minutes. The volume was then brought up to 1.5 ml with chilled veronal buffer, centrifuged at 2000 rpm for 5 minutes and readings taken. Titration end points were defined on the basis of 55% or less hemolysis. The MEFl and GDVII antigens were used interchangeably as controls and in a number of tests infant mouse adapted dengue virus(12) was also used as control antigen. Infectivity titrations were made in 3 to 4 weeks old mice using 10-fold

TABLE II. MEFl Complement Fixing Activity of Supernatant and Sediment Fractions Obtained by Centrifugation of Acetone-Ether Antigen at 35000 RPM for 75 Min.

Antigen	Infectivity	Complement fixation* Antigen titer	Serum titer
Saline-ether			
Control	$10^{-5.4}$	1:12	1:4096
Supernatant (¾ vol)	$10^{-2.4}$	1:1	1:16
Sediment (pellet and bottom ¼ supernatant)	$10^{-5.8}$	1:6	1:4096
Acetone-ether			
Control	$10^{-3.7}$	1:32	1:4096
Supernatant			
Upper ½ vol		1:8	1:1024
Lower ½ "		1:24	1:4096
¾ "	$10^{-1.6}$	1:16	1:4096
Sediment			
Pellet		1:32	1:4096
Pellet and bottom ¼ supernatant	$10^{-4.4}$	1:32	1:4096

* Anti-Lansing monkey serum 95305.
Blank = No test.

dilutions and the ID_{50} estimated by the method of moving averages(13).

Results. Table I illustrates the relationships of CF activity between saline-ether and acetone-ether extract antigens of MEFl virus. Although comparable serum titers were obtained with both antigens and hyperimmune sera, the acetone-ether antigens were of greater potency as evidenced by the 4-fold and higher antigen titers. The saline-ether antigen differed further in that it failed to give any significant reaction in human serum from a poliomyelitis patient infected with type 2 virus. It also failed to react significantly with sera from 3 additional patients (type 1 infection) which gave serum titers of 1:64 to 1:128 with the acetone-ether antigen. On the other hand the infectivity titer of the acetone-ether antigens was 6% and less that of the saline-ether antigens.

The effect of high speed centrifugation on the CF activities of both antigens was of much greater interest. Supernatants of acetone-ether extracts obtained by centrifugation at 35000 rpm (average g = 80730) in a Spinco Model L Ultracentrifuge had antigen titers 25 to 50% that of the controls even though 99% of the infectivity was removed and the sediment resuspended to volume was comparable to the controls in both CF activity and infectivity. In contrast, supernatants of saline-ether antigens were without or had only slight CF activity. Table II summarizes such an experiment. The sediment fraction of the acetone-ether antigens consistently gave stronger reactions than the supernatant fraction. This was evident not only on the basis of antigen titers, but also by the fact that the sediment gave slight but definite optimal ratio effects which was not obtained with the supernatant fraction (Table III).

The GDVII saline-ether antigen, in contrast to the same type of preparation of MEFl virus, had marked CF activity following centrifugation which removed over 99% of infectivity and hemagglutinating activity† (Table IV); and acetone-ether extraction resulted in a 16

TABLE III. "Box" Titration of 35000 Supernatant and Sediment Fractions of MEFl Acetone-Ether Antigen.

Antigen	Dilution	% hemolysis serum dilutions† 1:16	1:32	1:64	1:128	1:256	1:512
Supernatant	1:1	0	2.2	11.7	*	41.3	77.9
	1:2	0	5.8	31.3	74.3	88.8	95.2
	1:4	0	15.2	44.4	73.1	85.7	92.8
	1:8	25.8	59.3	80.7	91.3	96.7	95.8
	1:16	92.5	93.2	92.5	94.4	98.3	
Sediment	1:1	0	0	0	0	35.8	90.8
	1:2	0	0	0	0	14.3	87.3
	1:4	2	0	.9	.3	4.8	72
	1:8	2.3	1.4	6.0	5.1	27.9	69.2
	1:16	16.8	26.9	38.3	57.3	64.7	89.6
	1:32	74.3	75.2	91.3	91	92.8	93.3

Supernatant = ¾ vol. Sediment = Pellet and bottom ¼ supernatant.
* Excess sheep cells.
† Anti-Lansing serum 65301.

TABLE IV. Effect of Centrifugation at 40000 RPM (Avg g = 105400) for 2 Hr on Infectivity and Hemagglutinating and Complement Fixing Activity of Saline-Ether Extract of Infant Mouse CNS Infected with GDVII Virus.

Control			40,000 supernatant			40,000 sediment		
Infectivity titer	HA titer*	CF antigen titer†	Infectivity titer	HA titer	CF antigen titer	Infectivity titer	HA titer	CF antigen titer
$10^{-7.0}$ or >	1:819,200	1:128	$10^{-4.0}$	1:800	1:64	$10^{-7.0}$ or >	1:819,200	1:128

* Hemagglutination titer: Final dilution of supernatant.
† Serum: anti-GDVII mouse pool 65205.

TABLE V. Comparison of Complement Fixing Activity of Control, 40000 Supernatant and Sediment Fractions of Saline-Ether and Acetone-Ether Extracts of GDVII Virus.

	Control		Supernatant		Sediment	
Antigen	Antigen titer	Serum* titer	Antigen titer	Serum titer	Antigen titer	Serum titer
Saline-ether	1:64	1:256	1:16	1:128	1:32	1:128
Acetone-ether	1:512	1:128	1:512	1:128	1:512	1:256

* Mouse serum pool 95304.

to 32-fold concentration in CF activity of the sediment and supernatant fractions (Table V).

Studies with sera from poliomyelitis patients revealed significant differences in the CF titers obtained with the supernatant and pellet fractions of MEFl acetone-ether antigens. Of further interest is the fact that paired sera from 10 of 11 patients from whom types 1 and 3 viruses were isolated, gave titers of less than 1:16 with the supernatant fraction even though in some instances the titers with the pellet fraction were as high as 1:128 to 1:256. On the other hand, either the "acute" or "convalescent" sera from 3 patients from whom type 2 virus was isolated, had titers of 1:32 to 1:128 with the supernatant fraction, as compared to maximum titers of 1:64 to 1:512 with the pellet fraction. Obviously the data is too limited for definite conclusions. Moreover, these differences in such non-hyperimmune sera may, in part, reflect differences in the relative CF potency of the supernatant and pellet fractions.

Discussion and summary. There are several possibilities which may account for complement fixing activity present in the supernatants of acetone-ether but not in saline-ether extracts of MEFl infant mouse virus obtained by centrifugation at 35000 rpm for 75 minutes. The acetone-ether extraction not only inactivates a large amount of infectivity, but at the same time decreases the density of such inactivated virus or breaks down a portion of the virus particles into smaller units. The CF activity of the supernatant, on the other hand, may represent a second antigen evident with acetone-ether but not saline-ether extracts because of concentration effects. In addition, it is possible that acetone-ether but not saline-ether treatment releases from the virus particles a second antigen. Finally, inhibitor substance(s) which partially interfere with CF activity of MEFl virus may be removed by acetone-ether treatment. With GDVII virus it would appear that a physical separation of 2 CF antigens was realized, one associated with infectivity and hemagglutination, and the other of smaller size with comparatively little or no infectivity and hemagglutinating activity. Studies are now in progress to determine whether the supernatant CF activity of MEFl acetone-ether extract antigen represents an antigen qualitatively different from the sediment fraction and also to obtain further evidence for the multiplicity of antigens in GDVII mouse encephalomyelitis virus.

† It should be noted that GDVII infant mouse virus gave 32- to 128-fold higher hemagglutinating titers and approximately 10-fold higher infectivity titers than adult mouse virus(14).

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Relationship of Ulcer Pain to pH and Motility of Stomach and Duodenum. (21148)

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The exact cause of the pain of peptic ulcer has been the subject of controversy for many years. Following Carlson's(1) description of contractions of the stomach synchronous with ulcer pain, this pain was related to motility until Palmer(2-4) found that, in patients with active peptic ulcer, the instillation of 0.5% hydrochloric acid by gastric tube reproduced the pain, and immediate relief followed aspiration. Palmer concluded that ulcer pain resulted from direct chemical irritation of pain fibers in the base of the ulcer crater. Bonney and Pickering(5) confirmed Palmer's work. Ruffin and coworkers(6) reported observations on 56 peptic ulcer patients given an acid barium mixture during fluoroscopic examination. When pain was produced, persistent spasm of either stomach or duodenum occurred. If no pain developed, normal gastric motility and emptying were seen although the acid barium mixture was actually demonstrated in the ulcer crater.

Rollins and Friedlander(7) recorded kymograph tracings of either gastric or duodenal motility in patients with duodenal ulcer while ulcer pain was produced by the intragastric injection of 0.5% HCl. The administration of hexamethonium decreased motility, but did not relieve the ulcer pain. Palmer(8) studied 26 ulcer patients known to have had a positive acid test. Administration of anticholinergic drugs failed to alter the pain response in 23. Dragstedt and coworkers(9) investigated 5 patients with duodenal ulcers and with positive acid tests. Following surgical division of the vagus nerves, intragastric administration of hydrochloric acid produced the characteristic pain response for a period of 5-6 days postoperatively.

In view of the conflicting findings reported, it was decided to make further observations on patients with peptic ulcer pain under conditions whereby simultaneous motility records and pH determinations were obtained from both stomach and duodenum.

Method. Patients selected for study had active but uncomplicated duodenal ulcers. A triple-lumen tube was passed under fluoroscopic control into the duodenum; one lumen was used for inflating a balloon which recorded duodenal motility; another was used for periodic aspiration of duodenal contents; and the third lumen was used for the administration of N/10 HCl. A double lumen tube was then introduced into the pyloric antrum under fluoroscopic control. One lumen was

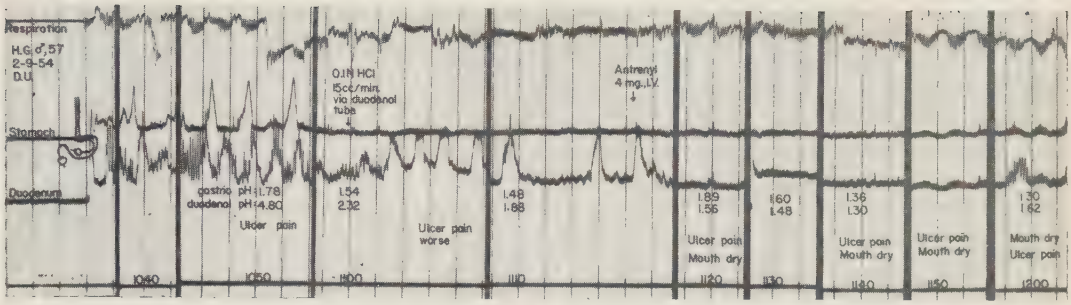

FIG. 1.

used for inflating a balloon which recorded gastric motility and the other was used for periodic aspiration of gastric contents. The gastric and duodenal motility were recorded on a multichannel recording oscillograph(10). The pH of gastric and duodenal aspirates was determined with a Beckman model G pH meter.

Results. I. 1-26-54. In a pilot study it was found necessary to introduce N/10 HCl into the duodenum at 180 drops/min. to reduce pH; ulcer pain did not occur until duodenal pH fell to 1.65.

II. On 2-9-54 gastric and duodenal motility and pH determinations were made on H.G., a 57-year-old white male (Fig. 1). Spontaneous ulcer pain was present initially; duodenal pH was 4.80, and gastric and duodenal activity were present. With the administration of N/10 HCl into the duodenum, duodenal pH dropped to 1.88 and ulcer pain was intensified. Duodenal motility continued unchanged; gastric motility ceased (as previously described by Quigley *et al.*(11) and others). Four mg of Antrenyl, Ciba (diethyl (2-hydroxyethyl) methylammonium bromide alpha-phenyl-cyclohexane-glycolate) was administered intravenously. Within 2 minutes all duodenal motor activity ceased for the duration of the study. However, duodenal pH remained between 1.30 and 1.62 and when pain persisted.

III. A.F., a 31-year-old Negro male was studied on 2-20-54. Initially the patient had only gastric motor activity, with a duodenal pH of 7.65. After the introduction of N/10 HCl, the duodenal pH dropped to 1.76 and simultaneously the patient experienced ulcer pain. Gastric activity ceased and duodenal motility began. Ten mg of Pathilon, Lederle (3 - diethylamino - 1 - cyclohexyl - 1 - phenyl-1-propanol ethiodide) was given intravenously. All motor activity of the duodenum ceased. The patient experienced slight momentary relief following which the pain markedly increased in intensity. The duodenal pH fluctuated between 1.31 and 1.40. When the HCl drip was stopped and normal saline was introduced into the duodenum, the duodenal pH gradually rose to 3.01 and the ulcer pain disappeared.

IV. H.T., a 47-year-old white male was studied on 2-27-54. Initially the patient had gastric and duodenal motor activity with a duodenal pH of 6.28. After the introduction of N/10 HCl for 10 minutes, the duodenal pH dropped to 1.55 and ulcer pain immediately began. Duodenal motility continued unchanged and gastric motor activity was reduced. The patient was given 30 mg of Probanthine, Searle (Beta-diisopropylaminoethyl xanthene-9-carboxylate methobromide) intravenously. Duodenal motor activity ceased for the duration of the study. However, ulcer pain persisted unabated. Because of the severity of the pain the HCl drip was stopped; at this point the duodenal pH was 1.40. Then 30 cc of an antacid was introduced into the duodenum and 7 minutes later duodenal aspiration started. Two minutes later duodenal pH was 4.02 and the patient was partially relieved; after 10 minutes the duodenal pH reached 6.02 and the patient was free of pain.

V. E.L., a 39-year-old white male was studied on 4-20-54. The patient displayed little gastric activity throughout the study. Intensive duodenal motility was present ini-

tially and continued after N/10 HCl drip was started. The patient experienced evanescent periods of mild distress; there was no consistent correlation between pain periods and duodenal contractions. Duodenal pH remained close to neutrality. Following the intravenous administration of 1.2 mg of atropine, duodenal contractions were markedly reduced in both frequency and amplitude. Six minutes after injection of atropine the duodenal pH declined to 1.68 and the patient noted moderately severe pain.

VI. In 3 additional patients with duodenal ulcer, the intraduodenal infusion of N/10 HCl inhibited gastric motility but failed to produce pain although the duodenal pH dropped to low levels.

Discussion. In most subjects, spontaneous gastric and duodenal motor activity were initially present. The intraduodenal administration of N/10 HCl invariably abolished gastric motility. It was found that surprisingly large volumes of N/10 HCl given at a rate of more than 180 drops/min. were required to drastically reduce the duodenal pH. For example, in one study 1300 cc of N/10 HCl was infused into the duodenum in a 60-minute period to reduce the duodenal pH to 1.68.

When the duodenal pH dropped below 1.88, pain of a persistent, burning nature occurred in 4 of 7 patients. The pain was always described as being characteristic of the patient's spontaneous ulcer pain. No change in the frequency or amplitude of duodenal contractions occurred with the onset of ulcer pain. This ulcer pain was not abolished by the anticholinergic drugs used, although duodenal motor activity was terminated. When the duodenal pH was elevated by neutralization or duodenal aspiration, pain was relieved in all cases.

Summary and conclusions. 1. In these experiments, ulcer pain was produced in 4 of 7 duodenal ulcer patients by the intraduodenal administration of N/10 HCl at a rate which lowered duodenal pH to 1.88 or less. 2. Onset of ulcer pain was not associated with any change in duodenal motility. 3. Ulcer pain induced by intraduodenal HCl was not relieved by anticholinergic drugs, even though duodenal motility was abolished. 4. When duodenal pH was elevated by neutralization or aspiration, ulcer pain was relieved. 5. Intraduodenal HCl constantly inhibited motility of the gastric antrum.

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A Facilitation Action of Reserpine on the Central Nervous System. (21149)

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Preparations from *Rauwolfia serpentina* have been used for centuries in Indian medicine for insomnia, hypochondria, and insanity (1). A sedative effect of these preparations was ascribed to be the rationale for therapy (2,3). Reserpine, the alkaloid isolated from *R. serpentina* recently, has been shown to possess a sedative property(4). In our studies of the influence of various agents on convulsive patterns in mice, reserpine was found to be capable of facilitating the extensor tonic component of the convulsion. This action is contrary to that of other central nervous system depressants which generally suppress the tonic extensor phase(5). The facilitation action of reserpine on the central nervous system appears to be specific in that it antagonizes selectively the anticonvulsant effects of the different depressants. This property of reserpine may be of diagnostic and therapeutic significance when applied to mental disorders.

In this report data are presented to show how reserpine affects the convulsive patterns induced in mice by chemical and electrical stimulation. The results obtained from experiments by antagonism between reserpine and the various anticonvulsant agents are to be reported in another communication.

Experimental. The reserpine, isolated from *R. serpentina* Benth. by Drs. Fleming and Crooks of the Research Laboratories, Parke, Davis & Co., was finely suspended in a gum acacia solution for intraperitoneal injection. Male albino mice weighing 18-22 g were used. They were allowed free access to food and water. Metrazol-, caffeine-, and strychnine-induced convulsions were produced by the intravenous infusion technic of Orloff, Williams, and Pfeiffer(6). Electrically-induced seizures were brought about in 2 ways: (a) with a low frequency stimulus similar to that employed by Toman(7), and (b) with a high frequency stimulus for maximal tonic convulsions as devised by Toman, Swinyard, and Goodman(8).

Results and discussion. The data in Table I indicate the influence of reserpine on seizure patterns of Metrazol-, caffeine-, and strychnine-induced convulsions. As described by Orloff, Williams, and Pfeiffer, Metrazol produces 3 "signs" of reaction which follow each other as the concentration of Metrazol is gradually increased in the blood stream. The first is a sharp twitching of the animal's body; this is followed very shortly by a series of clonic movements. After a period of alternate clonic movements and resting phases, the third sign, "persistent convulsion," occurs. It consists of a tonic flexion followed immediately by a tonic extension of the limbs. In the results here presented, only 2 endpoints were chosen for the seizure patterns by Metrazol: *viz.* the first sign of clonic movements and the final tonic extension component of the convulsion. Caffeine-induced convulsions are predominantly tonic seizures. Some clonic movements occur just before the tonic extension of the fore limbs. This, in a brief interval, is followed by the extension of hind limbs and usually death. The appearance of clonic movements was taken as one endpoint in our experiments. The full tonic extension of the hind limbs is the endpoint of injection. With strychnine, the mouse undergoes merely a short flexor and long extensor seizure of the limbs without eliciting any other premonitory signs as in the case of Metrazol and caffeine.

The effect of reserpine on Metrazol-induced seizures is a more rapid onset of the tonic phase of the convulsion. The final maximal extension of the limbs follows immediately the first sign of clonic movements. In other words, under the influence of reserpine, Metrazol will produce a seizure pattern in mice similar to that produced by strychnine. This is indicated by the fact that the same quantity of Metrazol employed for inducing the first clonic movements in untreated mice will produce a tonic extension seizure in reserpine-treated animals.

Reserpine exerted a similar effect on mice

TABLE I. Influence of Reserpine on Seizure Patterns of Mice Receiving Intravenous Infusion of Metrazol, Caffeine or Strychnine at Rate of 0.05 cc/10 Sec. 10 mice in each group.

Treatment	Metrazol (0.5%)			Caffeine (1.0%)			Strychnine (0.005%)	
	Wt, g	F.C.,* cc	T.E.,* cc	Wt, g	F.C., cc	T.E., cc	Wt, g	T.E., cc
Control	18-22	.14±.01	.44±.02	18-22	.29±.03	.36±.02	19-22	.34±.02
Reserpine, 8 mg/kg IP, 4-6 hr	19-22	.15±.00	.16±.01	18-20		.18±.02	18-22	.33±.01

* F.C., first clonic movements; T.E., tonic extension of hind limbs; mean value of cc solution and stand. errors.

with caffeine-induced seizure patterns. Here the amount of caffeine required for a maximal tonic extensor seizure in reserpine-treated mice is considerably less than that required for the first sign of clonic movements in untreated animals.

On the other hand, reserpine did not alter the strychnine-induced seizure patterns nor the dosage required for maximal tonic extensor response. That reserpine is capable of facilitating the tonic extensor seizure response in mice to a convulsive dose of Metrazol or caffeine but not to that of strychnine suggests that its site of action may be somewhere at the regions or pathways where both Metrazol and caffeine exert their analeptic action on the central nervous system.

This central effect of reserpine is slow in onset and long in duration. The dose and effect of reserpine at different periods after administration in Metrazol-induced seizures are given in Table II. No significant facilitation effect was evident 2 hours after the intraperitoneal injection of 4 mg/kg reserpine (section A). This is shown either by the quantity of Metrazol required for inducing tonic extensor seizures or by the number of mice in which tonic extensor response followed the first clonic movements immediately. Only half of the animals were affected at the third or fourth hour. An intraperitoneal dose of 8 mg/kg of reserpine for a period of 4 hours was found to produce a full effect in all animals (section B). This dose and the time interval were subsequently employed in other experiments for its maximal effect.

The prolonged effect of reserpine is indicated by the data in section C. A facilitated tonic extensor response was observed in mice as long as 6 days after 2 mg/kg of reserpine had been administered intraperitoneally.

In Table III are presented the results obtained from experiments in mice receiving electrical stimuli. With a current of 50-100 m.a., rectangular pulses of 6 per second frequency, and 1 msec. width, a "stunning" seizure response was induced in animals without medication. In this condition the mouse assumes an upright position without extension of the limbs. Under the influence of 8 mg/kg reserpine (4 hours after intraperitoneal injection), stimuli of equal magnitude produced a maximal tonic extension of the limbs in all animals. Half the reserpine-treated mice gave a maximal tonic extensor response to 24 milliamperes. For a tonic extensor response in untreated mice, either a higher frequency or a greater amperage of current was required. In this experiment, a current of 88 milliamperes and 9 per second frequency was found to produce a tonic extension of the hind limbs in 50% of the untreated animals.

In the case of maximal tonic extensor seizures induced in mice by a 60-cycle alternating current, the threshold stimulus was found to be markedly lowered by reserpine. The results in Table IV were obtained in an experiment in which the threshold stimuli were determined for mice 24 hours after various doses of reserpine were administered intraperitoneally. Each dose of the drug was given to 30 animals. They were divided into 3 groups of 10 mice each and shocked with 3 different current intensities which would produce a maximal tonic extensor response in from 10 to 90% of the test animals. The current threshold that would cause convulsions in 50% of the mice (CS_{50}) was estimated graphically by probit units and current intensities in logarithms(9). The data indicate that the threshold stimulus for mice was significantly lowered by 0.25 mg/kg of

TABLE II. Dose and Effect of Reserpine in Metrazol-Induced Seizures by Intravenous Infusion in Mice (0.5%, 0.05 cc/10 Sec). 10 mice in each group.

(A) Time of onset of reserpine effect, 4 mg/kg intraperitoneally

Time after inj. (hr)	Wt, g	Seizures F.C., cc	Seizures T.E., cc	Seizures (T.E. – F.C.) cc ± S.E.	Diff. in (T.E. – F.C.) after inj. cc	"t"	"P"	No. mice, F.C.→T.E.*
0	19-21	.15	.44	.30 ± .03				0
1	19-22	.16	.43	.27 ± .09	.03	1.4	>.1	1
2	19-22	.14	.38	.24 ± .04	.06	1.0	>.1	1
3	19-21	.15	.28	.13 ± .04	.16	3.1	<.01	5
4	18-20	.15	.26	.11 ± .04	.18	3.3	<.01	6

(B) Effect of reserpine at various dose levels, 4 hr after intraperitoneal inj. 10 mice in each group.

Dose, mg/kg	Wt, g	Seizures F.C., cc	Seizures T.E., cc	Seizures (T.E. – F.C.) cc ± S.E.	Diff. in (T.E. – F.C.) for dose given cc	"t"	"P"	No. mice, F.C.→T.E.*
0	18-21	.14	.44	.30 ± .03				0
1	19-22	.16	.48	.32 ± .02	.02	.5	>.1	0
2	19-22	.13	.37	.24 ± .05	.06	1.1	>.1	2
4	19-22	.15	.25	.10 ± .04	.20	3.7	<.01	5
6	19-22	.14	.23	.09 ± .04	.21	3.9	<.01	6
8	20-22	.15	.16	.01 ± .01	.29	8.2	<.01	10

(C) Duration of reserpine effect, 2 mg/kg intraperitoneally

Days after inj.	1	2	3	4	5	6	7	8	9	10
No. mice, F.C.→T.E.*	3/3	3/3	2/3	3/3	3/3	2/3	1/3	0/3	0/3	0/3

* F.C.→T.E. = Tonic extensor seizures followed first clonic movements immediately.

reserpine. Only above 1 mg/kg did the animals show the other symptomatic effects of reserpine, *viz.*, ptosis, depression, and diarrhea. The lowering of a maximal tonic extensor seizure threshold of current by reserpine had also been observed by Jenny at 40 mg/kg orally(10). In our experience reserpine is nearly 10 times more effective intraperitoneally than orally.

In view of the fact that Dilantin specifically suppresses the maximal tonic extensor seizures of mice under electroshock(5), the influence of reserpine on the anticonvulsant effect of Dilantin was investigated as follows: Thirty mice received 5 mg/kg of reserpine and 15 mg/kg of Dilantin intraperitoneally; another 30 animals as controls received the same amount of Dilantin only. The electroshock procedure was conducted as in the above experiment 6 hours after reserpine and 3 hours after Dilantin administration. The CS_{50} of the Dilantin and reserpine-treated mice and that of the Dilantin-treated controls were found to be 11.2 ± 1.2 and 51.5 ± 10.3 m.a., respectively, giving a significant difference of 30.3 ± 7.0 m.a.(11). Reserpine thus antagonizes the anticonvulsant effect of Dilantin. As will be discussed together for other anticonvulsant agents in another communication, the reaction between reserpine and Dilantin seems to be competitive in that the effect of one may be reversibly antagonized by that of the other.

The mode of this facilitation action of reserpine on the central nervous system awaits investigation. It may be that reserpine causes an increase in excitability or in conductivity of certain central nervous system structures. Some of its beneficial effects in mental patients may be due to this property. The evidence here presented points out that a precaution should be taken when using reserpine in epileptic patients to guard against possible precipitation of seizure attacks by the same mechanism. Reserpine may serve, on the other hand, as an activating agent to bring on

epileptic discharges electroencephalographically. Some caution should be taken also in reserpine-treated patients on convulsive therapy either by chemical or by electrical stimulation. The dosage of a convulsant such as Metrazol or the current intensity required will be less, while the tonic extensor phase of the convulsion is expected to be facilitated under the influence of reserpine.

Summary. 1. Data are presented to show a facilitation action of reserpine on the central nervous system. The drug hastens the onset of maximal tonic extensor seizures in mice receiving intravenous Metrazol or caffeine (but not strychnine). It lowers the convulsive seizure threshold of mice to electrical stimuli and antagonizes the anticonvulsant action of Dilantin. 2. Some possible mechanisms of action and certain clinical implications of this property of reserpine are suggested.

TABLE III. Influence of Reserpine on Seizure Patterns of Mice Induced by a Low Frequency Rectangular Pulse Current.

Treatment, mg/kg I.P.	Current Cycles /sec.	m.a.	"Stunning" response No. reacted / No. used	Tonic extension No. reacted / No. used
0	6	25	10/10	0/10
0	"	50	10/10	0/10
0	"	100	10/10	0/10
Reserpine, 8*	"	10	5/5	0/5
	"	15	5/5	0/5
	"	20	7/10	3/10
	"	25	4/10	6/10
	"	35	1/10	9/10
	"	50	—	10/10
	"	100	—	10/10
0	9	50	9/10	1/10
0	"	75	6/10	4/10
0	"	100	5/10	5/10
0	12	100	—	10/10

T.E.$_{50}$† reserpine = 24 ± 1.4 m.a., 6 ~
T.E.$_{50}$ untreated = 88 ± 7.9 m.a., 9 ~

* 4.5 hr after inj.
† Tonic extension seizures in 50% of animals.

TABLE IV. Effect of Reserpine on Maximal Tonic Extensor Seizures in Mice Induced by a 60 Cycle Alternating Current for 0.2 sec. Thirty mice in each group.

Dose of reserpine, mg/kg IP (24 hr)	CS_{50},* m.a. ± S.E.	Difference† in CS_{50}	Significance "t"	Significance "P"
0	5.09 ± .10			
.0125	4.90 ± .09	.19	1.40	>.1
.025	4.80 ± .09	.29	2.10	.05
.05	4.71 ± .11	.38	2.50	.02
.10	4.72 ± .11	.37	2.40	.02
.25	4.70 ± .07	.39	3.10	<.01
.50	4.53 ± .07	.56	4.50	"
1.00	4.26 ± .07	.83	5.90	"
2.00	3.55 ± .06	1.54	11.30	"
4.00	3.10 ± .05	1.99	16.00	"

* CS_{50}—Convulsive stimulus for 50% of mice.
† Difference in convulsive stimuli required for untreated controls and for reserpine treated animals.

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Contribution to the Pharmacology of Apoatropine and Its Methyl Bromide. (21150)

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Apoatropine is found to a limited extent in belladonna root(1). It is an anhydride of atropine (with respect to the tropic acid moiety of the molecule) and is prepared from atropine by the action of dehydrating agents (2). There are several fragmentary studies of its pharmacology recorded during the past half-century. Apoatropine differs from atropine in that it elicits only a mild mydriatic response(3). It was found that in larger doses it evokes increased respiratory activity and finally convulsive seizures. Our interest in this compound stems from the studies of Kreitmair and Wolfes(4), who observed that apoatropine was 5 times more potent than atropine in relaxing a barium ion-induced spasm in the isolated intestinal strip of the rabbit. These investigators confirmed the previously-mentioned action of apoatropine on the central nervous system. They also observed that its acute toxicity to mice was 20 times greater than that of atropine. We considered it interesting to study apoatropine as an antispasmodic and also to compare it with its methyl bromide, which to our knowledge has never been compared.

Materials. The apoatropine used in these studies was prepared for us by Dr. Verne C. Bidlack, Jr.,* by dehydrating atropine with sulfuric acid. The compound melted between 256 and 257°C. The hydrochloride was found to contain 11.72% chlorine (theory 11.85%). Apoatropine methyl bromide prepared from this sample of apoatropine melted between 253 and 256°C.

Acute toxicity. Upon intraperitoneal injection in the mouse the LD_{50} for apoatropine was 14.1 mg/kg with 95% confidence limits of 17.5 mg/kg and 11.4 mg/kg. Under the same conditions the values reported for atropine in the literature are between 200 and 300 mg/kg. Apoatropine was much less toxic to the mouse when administered orally. The LD_{50} orally was found to be 160 mg/kg with 95% confidence limits of 115.1 mg/kg and 222.4 mg/kg.

TABLE I. Oral Administration of Apoatropine to 4 Dogs.

Wt, kg	Dose, mg/kg	24 hr	48 hr	72 hr
14.2	40	X and M*	Recovery	
12.1	80	*Idem*	Dead	
9.6	160	" & emesis		Dead
5.4	320	*Idem* with convulsions	Recovery	

* X and M = Xerostomia and mydriasis.

Chronic toxicity. Apoatropine was given by stomach tube to mongrel dogs weighing between 6 and 10 kg. The effect and dosage levels are given in Table I.

Three groups of 6 rats were given apoatropine orally each day for 5 days at dosage levels of 20, 40 and 80 mg/kg. Each animal appeared to tolerate the drug without symptoms. Weight gains were not retarded and none of the animals succumbed to the treatment.

Circulatory effects of apoatropine. Apoatropine was injected intravenously into 3 dogs anesthetized with ether. Blood pressure recordings and electrocardiograms were made.

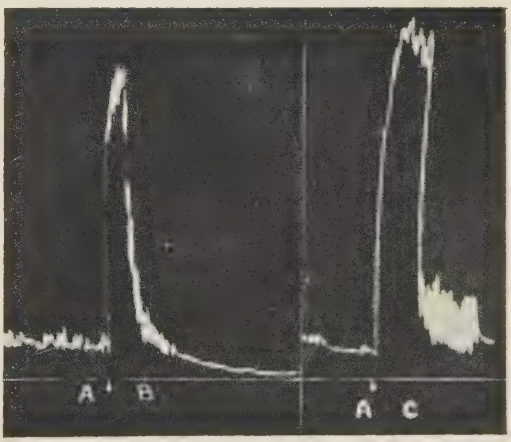

FIG. 1. Action of apoatropine and atropine on the isolated strip of the rat's intestine. L. Apoatropine. R. Atropine. A. 1 mg barium chloride. B. 0.5 mg apoatropine. C. 1 mg atropine. Volume of bath 30 ml.

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TABLE II. Passage of Charcoal Mixture.

Drugs administered	No. of rats	% of intestine through which charcoal passed	P value
Control	8	81.3	
Atropine sulfate, 0.5 mg/kg	5	50.0	<0.01
Apoatropine hydrochloride, 20 mg/kg	10	41.2	<0.01

In dosage levels from 2 to 5 mg/kg the following findings were observed. A precipitous fall in blood pressure occurred immediately upon injection. There was a concomitant bradycardia and depression of the rate and amplitude of respiration. Mydriasis was present. The cardiac vagus was blocked to faradic stimulation. The bradycardia was progressive. In one animal in which death occurred the respiration continued for a short period after the cessation of the heart beat. The electrocardiographic findings were depression of the R-spike, depression of the heart rate and increase in the P-R interval.

Intestinal motility of the rat in vitro. Our principal interest in apoatropine stemmed from its possible availability as an anti-spasmodic. On smooth muscle we were able to confirm the work of Kreitmair and Wolfes(4) in that the antispasmodic action was similar to that of atropine. With 7 strips of rat's intestine, the rat's uterus and rabbit's intestine, our findings indicate that atropine and apoatropine evoke about the same degree of antispasmodic action. The antispasmodic action of apoatropine obliterated most of the rhythmic contractions of the muscle. The action of atropine, however, invariably was followed by diminished rhythmic contractions. The respective antispasmodic actions of the 2 compounds are shown in Fig. 1.

Intestinal motility of the rat in situ. Apoatropine and atropine were compared on the intestinal motility of the rat by the method of Van Liere(5). The method depends upon the relative distance traveled by a charcoal acacia mixture from the pyloris along the intestine in a period of 40 minutes after its oral administration. The drugs are administered intraperitoneally 10 minutes prior to the charcoal mixture. The data are shown in Table II. This dosage of apoatropine produced convulsive seizures in half of the animals. The experiment was repeated using 5 rats at a 2.5 mg/kg dosage level. No diminution of the rate of intestinal propulsion was observed.

Apoatropine methyl bromide. By quaternizing the nitrogen atom in homatropine the useful antispasmodic and less toxic homatropine methyl bromide is formed. Accordingly we studied the corresponding quaternized apoatropine. Toxicity was enhanced. The intraperitoneal LD_{50} in the mouse was 0.76 mg/kg with confidence limits of 1.36 mg/kg and 0.42 mg/kg. Employing approximately this dose in the rat, 0.7 mg/kg, in 8 rats the intestinal motility was reduced to 58%. Many of the animals manifested toxic symptoms. It is obvious that by quaternizing apoatropine one enhances the toxicity approximately 20-fold, with no widening of the margin of safety as an antispasmodic as measured by the action on the rat's intestine.

Summary and conclusions. 1. Apoatropine is about 20 times more acutely toxic than atropine. 2. On the isolated rat's intestine atropine and apoatropine elicit about the same degree of antispasmodic action. 3. In the intact rat, apoatropine is approximately one-fortieth as active as atropine in producing the tranquilization of the small intestine. 4. Apoatropine is a potent cardiac depressant in the dog. 5. By quaternizing apoatropine one enhances markedly its toxicity without appreciable effect upon its antispasmodic action.

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Serum Lipid Concentration in Blood from Afferent and Efferent Pulmonary Vessels in Man. (21151)

ALVAR SVANBORG. (Introduced by D. D. Van Slyke.)

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In experiments on dogs Abelous and Soula (1) observed that serum from the right heart had a higher cholesterol content than serum from the left heart. Furthermore, they found an autolytic decrease in the cholesterol content in extirpated lung tissue that had been protected from bacterial decomposition under storage. In their opinion these observations indicated that breakdown of cholesterol takes place normally in the lung tissue. Bouisset and Soula(2) demonstrated that the decrease in serum cholesterol in the passage through the lungs increased during hyperventilation. Since that time numerous writers have reported similar results. A review of the literature may be found in Shillito *et al.*(3). Bugnard(4) believed he could verify the observations of earlier investigators of a decrease in the cholesterol concentration in serum in passage through the lungs, but contended that this decrease was due to a migration of cholesterol into the blood corpuscles. This cholesterol migration from the serum to blood corpuscles he considered regulated by changes in the pH of blood (Bugnard(4)). Other investigators have reported contradictory results. Markowitz and Mann(5) were unable even after increased fat administration to demonstrate differences in the total lipid concentration in afferent and efferent pulmonary vessels in dogs. Shillito *et al.*(3) reported similar results in serum cholesterol investigations in dogs.

Determinations of the lipid concentration in serum from the right heart have not heretofore been carried out in man. Such an investigation is reported in the present work. Blood was collected for analysis from the brachial artery by puncture and from the pulmonary artery at heart catheterization. The patients studied exhibited no signs of pulmonary disease. Two patients were investigated in conjunction with abdominal operations. One patient was catheterized because of suspected heart disease, but the findings at the examination were normal. Six patients had heart diseases; 4 of these without right-to-left shunt. For comparison 2 cases of heart disease with right-to-left shunt were included. The diagnoses appear in the table.

Methods. Total lipid content determination. The serum was dropped into absolute alcohol, heated to just under the boiling point. The precipitate was removed by filtration and Soxhlet extracted with absolute alcohol for 5 hours. The alcohol was evaporated, and the lipids were redissolved in chloroform. From several portions of this chloroform solution, the lipids were determined by means of evaporation and weighing of the residue. Cholesterol and lipid phosphorus concentrations were determined according to methods described in an earlier publication (Svanborg(6)).

Results. The results are presented in the table. No tendency to a decrease in lipid concentration in the passage of blood through the lungs was demonstrated. The differences obtained lie completely within the limits of error of the method.

Discussion. It has been shown repeatedly in *in vitro* experiments that lung tissue is able to break down lipids (Falk(7); Geyer, *et al.* (8); Alfin-Slater, *et al.*(9); Meyer, *et al.* (10)). It is impossible, however, to draw definite conclusions from these *in vitro* experiments concerning the magnitude of the lipid breakdown in the lungs *in vivo*.

As pointed out in an earlier work (Svanborg(6)), it is frequently impossible to assess the quantitative enzyme activity of an organ solely by determination of differences in substrate concentration in blood from afferent and efferent vessels. It is necessary to take into consideration the possibility of enzyme activity of opposite effect in the organ, the possible accumulation of substrate in the

TABLE I. Total Lipid Cholesterol and Lipidphosphorus Concentration in Blood from Pulmonary Vessels.

Case	Pulmonary artery			Brachial artery			Diagnosis
	Cholesterol, mg/100 ml	Lipid P, mg/100 ml	Total lipids, mg/100 ml	Cholesterol, mg/100 ml	Lipid P, mg/100 ml	Total lipids, mg/100 ml	
H.N.	200	5.9	590	190	5.9	670	Peptic ulcer
P.L.	300	11.3	1250	330	11.1	1080	" "
R.P.	220	6.1	890	200	5.6	950	Normal case
J.S.	200		790	210		680	Ess. pulm. hypertension
E.K.	180	5.4	810	210	6.2	830	Pulm. artery stenosis
P.M.	200	6.9	770	190	6.5	690	Mitral valve disease
E.A.	220	7.1	880	220	7.7	1170	*Idem*
K.E.	180	7.0	650	160	7.5	630	Pat. duct. art.
S.M.	180	5.3	460	180	5.4	580	Atr. sept. defect

organ, the loss of substrate through lymph circulation, and mucosal secretion, among other factors.

It is important to establish that even if an appreciable lipid breakdown normally occurred in the lungs, the decrease in lipid concentration in blood in the passage through the lungs would be small since the rate of circulation through the lungs is extremely high. Actually, it may be estimated that this decrease in concentration cannot conceivably be sufficient to permit demonstration with the available methods of lipid concentration determination.

Consequently, the results presented here do not preclude the possibility that a lipid breakdown normally takes place in the lung tissue.

Summary. 1. Numerous earlier investigators have maintained that they demonstrated a lower serum cholesterol concentration in blood from efferent than from afferent pulmonary vessels in experimental animals. These observations have been interpreted as indications of cholesterol breakdown in lung tissue. 2. Cholesterol, lipid phosphorus and total lipid concentrations were determined in blood from the left heart (brachial artery) and the right heart (pulmonary artery) in 9 patients. No decrease in lipid concentration in serum in the passage of blood through the lungs was demonstrated. 3. The difficulties in attempting to evaluate the quantitative enzyme activity of an organ solely by determination of the substrate concentration in afferent and efferent blood vessels is discussed.

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Application of Manometric Method to Testing Chemical Agents *in vitro* for Interference with Poliomyelitis Virus Synthesis.*† (21152)

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The need for a reliable *in vitro* screening procedure to expedite the search for a chemical inhibitor(s) of mammalian virus infection has led to a variety of approaches to this problem. Advantage may be taken of the observation that a human epithelial cancer cell, strain HeLa, and any of the 3 types of poliomyelitis virus possess attributes which provide in combination a model cell-virus system for quantitative studies(1-3). Thus, poliomyelitis virus excels as a test agent since it is stable under widely different conditions, and it can be readily handled and accurately quantitated. Similarly, the HeLa cell strain in continuous culture has extraordinary properties: It is a) stable, b) yields cells on a 2-dimensional surface such as glass for uniform exposure to virus, c) provides a cell population that can be accurately measured, and d) upon infection by a cytopathogenic virus responds by rapidly progressive total destruction. Direct observation for cytopathology permits recognition of any interference of viral infection that may result from a test chemical inhibitor. These properties of host cell and of virus make it possible to study a test material for an alterative effect upon the host cell, or upon virus synthesis, or both. A relationship of virus synthesis to actively metabolizing cells is recognized. For example, other workers have related the dependence of virus synthesis on oxidative energy of cellular respiration for the influenza-embryonic chick system(4), for the influenza-chorioallantoic membrane system (5), and for the feline pneumonitis-yolk sac system(6). If a similar relationship were to hold for the synthesis of poliomyelitis virus by strain HeLa cells, it would render suspect the usefulness of a chemical that reduces concurrently synthesis *in vitro* and the respiratory rate of the host cell. Moreover, the respiration of the surviving cells reflects the progress of infection by a cytopathogenic virus. The present study reports findings of manometric studies which employed cultures of strain HeLa cells to test the effect of antimetabolites upon poliomyelitis infection. These studies demonstrate that the chemical agents employed can influence the replication of poliomyelitis virus only when used in a concentration that inhibits markedly cellular respiration.

Materials and methods. Microbial contamination was avoided by aseptic technic and the employment of penicillin and streptomycin in culture medium at levels of 300 units per ml and 100 μg per ml respectively.

Assay for virus. Virus titrations were conducted essentially as described(1,2) with the following exceptions: a) cell cultures in preparation for incubation were rinsed thrice instead of twice; b) 5 replicate tubes were set up at each successive geometric dilution so to permit by use of standard statistical tables computation of most probable numbers of tissue culture infectious doses (TCID). Final virus dilutions were made in a solution consisting of 1 part chicken serum to 9 parts of maintenance solution; 1.0 ml of each dilution was added per tube. The reproducibility of such titrations in this laboratory has been found to be of the order of ± 0.3 log. *Virus*: Poliomyelitis virus, Type 1, Mahoney strain was employed. The immediate source of virus was a pool of supernatant fluids from cultures representative of the 63rd passage of the virus in strain HeLa cells. Virus suspensions were stored in 2 ml glass ampules at $-70°$C. A small amount of virus (15-30 TCID) was employed in each experiment so that several cycles of virus synthesis could be followed. *Host cell:* Strain HeLa cells were employed,

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cultivated, maintained, and enumerated as described previously(1-3). Cells were grown in planar bottles with a nutritive medium composed of 40% human adult serum, 2% chicken embryonic extract, and 58% Hanks' balanced salt solution (HAS-40, EE-2, BSS-58).

Metabolic analogues. The metabolic analogues, DL-ethionine,§ benzimidazole,§ β-2-thienylalanine,‖ 2,6-diaminopurine,‖ and the metabolite DL-methionine§ were used as isotonic solutions prepared by dilution with BSS to a final ionic concentration of 0.33 M. All solutions were sterilized by filtration through ultrafine sintered glass filters.

Phosphite buffer-salt solution (BPS). A sodium hydrogen phosphite buffer¶ replaced MS for experiments in which additional buffering capacity was desired. A 0.20 M stock solution of H_3PO_3 was prepared from Baker's purified 30% solution and checked acidimetrically. 100 ml of this stock, adjusted to pH 7.6 with NaOH, and diluted to 200 ml with distilled water, yielded an isotonic 0.1 M solution of NaH_2PO_3-Na_2HPO_3 of pH 7.6. This solution was sterilized by passage through an ultrafine sintered glass filter. Phosphite anion has been found(7) to be a relatively non-toxic buffer with an useful buffering range of pH 5.5 to 7.5. It is compatible with calcium and magnesium salts, does not interfere in known enzymic reactions of phosphate, and when present at 0.02 M concentration in the medium, does not reduce the growth rate of strain HeLa cells in stationary culture nor reduce the rate capacity of the cell to synthesize poliomyelitis virus.

Manometric methods. The Krebs' modification of Pardee's carbon dioxide buffer(8) was employed in respiration studies at an equilibrium concentration of 2% carbon dioxide in the gas phase. Carbon dioxide buffer, 0.5 ml, was placed in the center well fitted with a filter paper wick and another 0.5 ml placed in the side arm.

A convenient rate of respiration was found with from 4 x 10^5 to 2 x 10^6 cells per flask. To obtain the requisite number of cells for each experiment, cultures of several bottles were trypsinized, pooled, concentrated by centrifugation at 160 g for 10 minutes, washed by resuspension in BSS to original volume and recentrifuged as before. Cells were suspended in a volume of MS-100 so that the desired number of cells would be contained in the 1 ml aliquots transferred to the main compartment of each sterile Warburg flask. Flasks then received 0.2 ml of ChS-100 and 0.7 ml of either BSS or test chemical in BSS. Cultures were infected by inoculation with 0.1 ml of virus suspension containing 15 to 30 TCID; control flasks received 0.1 ml of BSS in lieu of above. To reduce the hazard of contamination, the ground glass connections of the manometers were sterilized by repeated immersion in 95% ethanol and ignition and then lubricated with sterile petrolatum. The sterile venting tubes fitted with absorbent cotton filters were connected to the gas supply and inserted in the flasks. The flasks were mounted on the manometers, immersed in the water bath maintained at 35°C and gas was passed through the system for 1 hour while the flasks were shaken at a rate of 60 strokes per minute and an amplitude of 2.5 cm. Stopcocks were then closed and the respiration followed in the conventional manner for a period of several days.

Experimental. Pilot studies. The first experiments were designed to test the thesis that the energy derived from respiration was essential for synthesis of poliomyelitis virus by strain HeLa. The capacity of HeLa cells to produce poliomyelitis virus, Type 1, under aerobic and anaerobic conditions was compared. The amount of acid produced by the HeLa cells under anaerobic conditions was greater than under aerobic conditions. To maintain a comparable pH in both systems, it was necessary to add additional buffer. The possibility of using phosphite buffer at 0.02 M concentration was tested by observing the effect on the rate of respiration of HeLa cells. Two series of Warburg flasks were prepared

§ Nutritional Biochemicals Corp.

‖ Bios Laboratories, Inc.

¶ 200 ml of 0.1 M phosphite solution at pH 7.6, 1.0 g glucose, 6.25 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.14 g $CaCl_2$, 0.35 g $NaHCO_3$, 0.06 g KH_2PO_4, 0.06 g Na_2HPO_4, and distilled water to make 1 liter. The final concentration of phosphite is 0.02 M.

TABLE I. Effect of Sodium Acid Phosphite (.02M) upon Respiration of Strain HeLa.

Hr	O_2 uptake, μl		
	MS* (control)	BPS† (test)	% of control
12	127	117	92
24	250	236	94
36	371	366	99
48	487	496	102
72	708	726	103

* Maintenance solution.
† Balanced phosphite solution.

with carbon dioxide buffer in the center well and side arm of each flask. Chicken serum, 0.2 ml, was added to the main compartment of each flask. HeLa cells, 1.1 million, suspended in either 1.8 ml of BPS or 1.8 ml of MS were added to each flask. The thermobarometer was prepared by adding a like amount of carbon dioxide buffer to the center well and side arm, and by placing medium without cells in the main compartment. Oxygen uptake was followed for 72 hours in the conventional manner. The results (Table I) indicated that 0.02 M phosphite did not affect the respiration of the HeLa cells.

Replicate Warburg flasks were prepared using BPS-90, ChS-10. An inoculum of from 20-30 TCID of virus in a volume of 0.2 ml of BPS was added to the side arm of each flask. The aerobic flasks were incubated in an atmosphere of 2% CO_2, 98% air with CO_2 buffer in the center well and side arm equilibrated in the usual manner. The anaerobic flasks were equilibrated in an atmosphere of 100% nitrogen (oxygen-free) with continuous shaking for 1 hour in the water bath. The contents of the side arm were dumped into the main compartment. The cells under anaerobic conditions remained viable, as evidenced by the production of carbon dioxide and progressive accumulation of acid. However, the virus yield from these cells was only 0.01% of the amount produced by an equal number of cells maintained aerobically. Thus, the anaerobic yield of virus in TCID/ml was $10^{3.1}$ as compared to an aerobic yield of $10^{7.2}$.

A comparative study of the respiratory rate and virus yield was made of cultures shaken continuously at 60 strokes per minute and an amplitude of 2.5 cm, and of cultures held stationary to learn whether the vigorous agitation imposed on HeLa cells in Warburg flasks might affect adversely their metabolic rate. If cellular respiration were affected markedly the results of such manometric studies might be difficult to relate to findings with stationary cultures. HeLa cells attach and grow readily on a glass surface; in agitated cultures they assume a position at the gas-liquid-glass interface. As shown by the data in Table II, the initial respiratory rate of agitated cultures was somewhat greater than that of static cultures, presumably from more rapid diffusion of oxygen through the medium. Nevertheless, the total O_2 uptake and virus synthesized were essentially unaltered by such agitation.

TABLE II. Effect of Agitation on Respiration and Virus Production by HeLa Cells.

	O_2 uptake, μl			Virus yield, log TCID/ml (44 hr)
	12 hr	24 hr	44 hr	
Shaken*	37	73	93	6.5
	38	74	92	6.7
Stationary	29	59	87	6.4
	32	62	92	6.5

* 60 strokes/min., amplitude of 2.5 cm.

Tests with metabolic analogues. The stationary tube method and the manometric method were compared for the determination of toxicity of benzimidazole for strain HeLa cells and for the resultant effect on virus yield. Parallel experiments were performed in duplicate Warburg flasks and in quintuplicate test tube cultures. Each Warburg flask was

TABLE III. Comparison of Virus Yield and Detection of Toxicity by Test Tube and Manometric Methods.

Benzimidazole, mg/ml	56 hr virus yield, log TCID/ml		Toxicity* to tube culture, 56 hr	Inhibition of respiration (%), 56 hr
	Manometric	Tube		
.0	7.0	3.9		
	7.0			
.25	6.4	3.0	—†	36
	6.5			
.50	3.7	1.4	+	80
	3.5			

* Microscopic evidence for toxicity in test tubes was assessed by contraction of cytoplasm, by granularity, and/or cells leaving the glass.
† Control tubes incubated for an additional 24 hr showed questionable evidence of toxicity by slight rounding of cells.

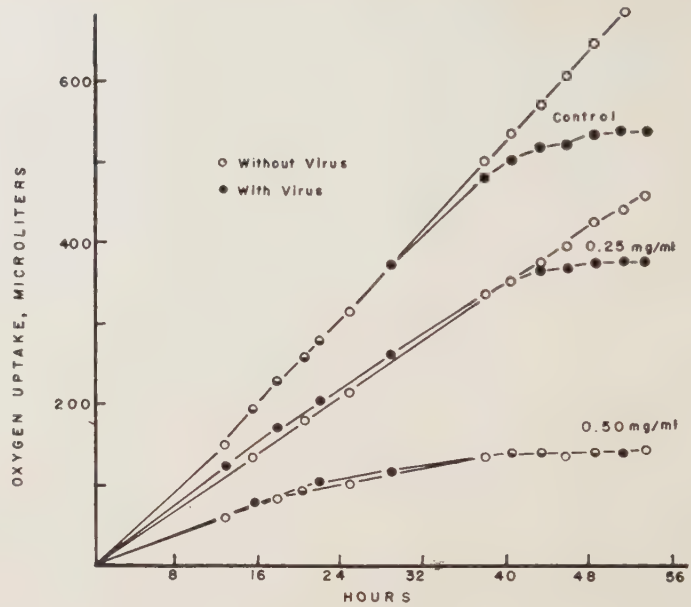

FIG. 1. Effect of benzimidazole upon respiration of strain HeLa.

charged with 5 x 10^5 cells; comparable tube cultures each contained approximately 5 x 10^4 cells. After 55 hours incubation at 35°C, the supernatant fluids were harvested separately and assayed for virus content. The results are given in Table III. The presence of 0.25 mg/ml of benzimidazole significantly depressed the virus yield in tube cultures over a period when control tubes showed no microscopic evidence of toxicity. However, this concentration of benzimidazole was found to markedly inhibit respiration. This interference with cellular respiration was apparent from the outset and progressive as shown in Fig. 1. Similar manometric studies were carried out with 3 other metabolic analogues, *viz.*, DL-ethionine, β-2-thienylalanine, and 2,6-diaminopurine. The results are summarized in Table IV. The effects of these antimetabolites on the respiration of HeLa cells are shown in Fig. 2 to 4. Supernatant fluids for virus titrations were removed shortly after respiration ceased. It was observed in each experiment that cellular death of infected cultures, as indicated by the cessation of respiration, was not influenced by the presence or absence of antimetabolite, or by the amount of virus produced. Thus again, significant inhibition of virus synthesis was limited to the concentrations of antimetabolite that provoked marked inhibition of the respiration of the host cell. Included in Table IV are the results of two attempts to reverse the toxic effects of ethionine. Methionine was able to partially reverse the inhibition of ethionine.

Discussion. DL-ethionine, 2,6-diaminopurine, benzimidazole, and β-2-thienylalanine did not inhibit the synthesis of poliomyelitis virus, Type 1, in strain HeLa cell cultures except at concentrations which were markedly inhibitory to the respiration of the host cell. Cessation of respiration of infected cultures occurred at the same time independent of the concentration of metabolic analogue employed, or of the final concentration of virus. The cytopathic changes reflected in reality the additive effects of chemical and virus. With the possible exception of benzimidazole, toxicity increased with duration of exposure to the antimetabolite. The respiratory inhibition provoked by ethionine was only partially reversed by the addition of methionine.

A concentration of benzimidazole, 0.25 mg/ml, which was non-toxic for HeLa cells as assessed by microscopic observations, was markedly inhibitory to the respiration of cells in a Warburg flask. This observation indicates that the detection of altered respiration

TABLE IV. Effect of Metabolic Analogues upon Respiration of Strain HeLa and upon Replication of Poliomyelitis Virus.

Exp. No.	Compound	mg/ml	% inhibition of respiration 24 hr	48 hr	72 hr	Virus yield, log TCID/ml	No. of cells per flask $\times 10^{-5}$
1.	Benzimidazole	0				7.0	5
		.25	32	34	38	6.4	
		.50	68	78	79	3.6	
2.	β-2-thienylalanine	0				6.5	5
		.10	0	0	0	6.6	
		.25	0	10	17	6.4	
		.50	9	24	33	5.8	
3.	2,6-diamino-purine	0				6.5	5
		.025	0	8	24	6.3	
		.050	4	13	30	6.8	
4.	2,6- " "	0				6.7	6
		.5	34	54	63	3.7	
5.	DL-ethionine	0				7.5	8
		.68	0	3	19	7.5	
6.	DL- "	0				7.3	12
		1.3	27	44	56	6.3	
		2.7	33	57	71	5.3	
	DL-ethionine + DL-methionine	1.3 + 1.2	23	37	42	6.6	
7.	Dl-ethionine	0				7.6	10
		2.7	33	56	73	6.3	
	DL-ethionine + DL-methionine	2.7 + 2.5	27	42	51	7.5	

might prove generally a more sensitive criterion of drug toxicity than the subjective observation of any alteration in morphology.

The importance of the oxidative energy of respiration for the synthesis of poliomyelitis virus, Type 1, by HeLa cells was demonstrated. Anaerobically, such cells were able to produce less than 0.01% of the amount of virus produced by replicate systems under aerobic conditions. This small anaerobic production of virus may reflect either stored energy reserves of the host cell or incomplete removal of the oxygen from the medium during equilibration.

The importance of oxidative energy of respiration for the synthesis of viruses in other virus-host systems has been reported by several investigators(4-6). If an agent so interfered with essential metabolism that it reduced the respiration of the host cell and hence the supply of energy available for endergonic processes, it would not be remarkable if virus synthesis was also suppressed. Such non-specific interference with virus synthesis in homogeneous cell populations could be recognized readily by observations of altered respiratory rate. Thus, the homogeneity of HeLa cells in culture makes this cell strain distinctly superior to any mixed cell culture, for in the latter, the proportion of cells susceptible to virus or to chemical agent is variable and may be small.

HeLa cells in each of the cultures here employed have been susceptible to infection and subsequent destruction by poliomyelitis virus. This evidence for cellular uniformity in susceptibility to the test virus permits one to assign the observed effects of added agents on respiration of such cultures to the effect of the agent on those cells capable of propagating the virus. Such a system of uniform cells can serve as an index of the progress of infection by cytopathogenic viruses. Presumably any agent capable of interference with virus synthesis without blocking essential metabolism of the host cell would behave by prolonging the respiration of infected cultures.

The desirability of interfering with virus synthesis *per se* rather than with essential metabolism of the host cell may not be realized

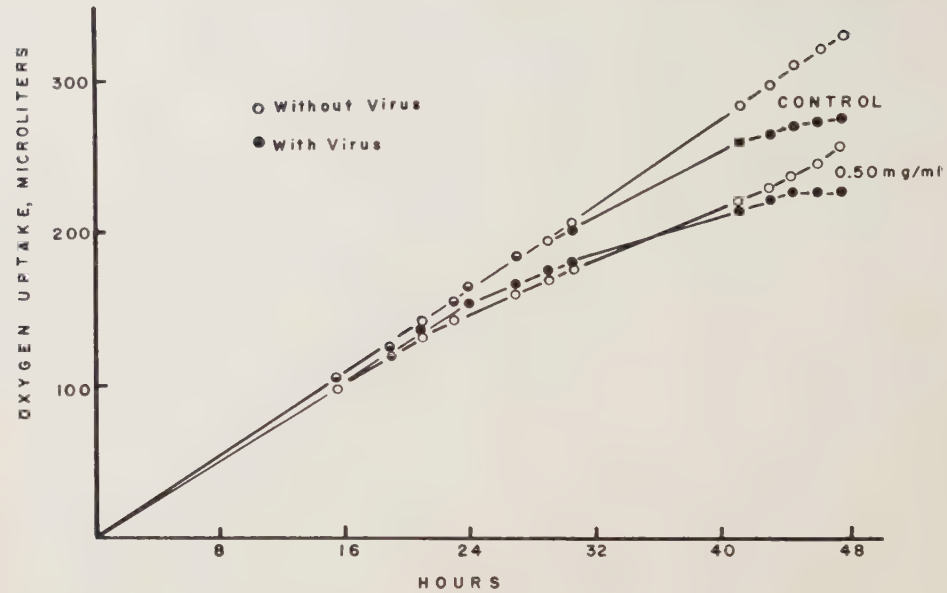

FIG. 2. Effect of β-2-thienylalanine upon respiration of strain HeLa.

without careful assessment of the toxicity of such chemicals. The demonstration of recovery of cells after removal of drug, or upon addition of metabolite, has been generally used in heterogeneous tissue culture to indicate the degree of permanent damage inflicted on the host cell by the test agent. More recently, cell cultures amenable to visual observations have permitted changes in morphology or growth rate to serve as a measure of toxicity of such added agents. Determination of toxicity by observation of the effect of added chemical agents on cell respiration has been employed in mixed cell cultures(5,9,10). Since the proportion of cells susceptible to virus or to chemical was not established by

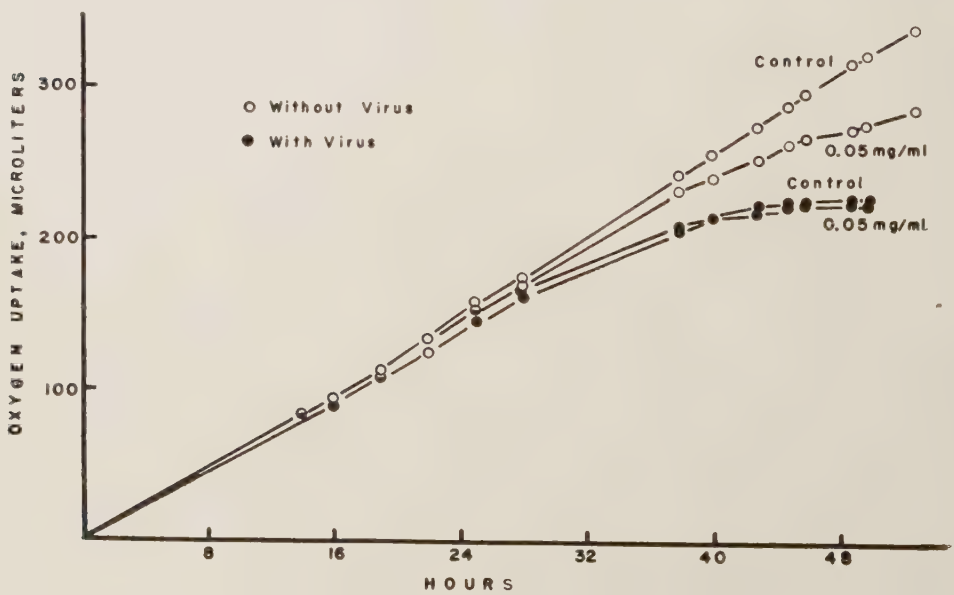

FIG. 3. Effect of 2,6-diaminopurine upon respiration of strain HeLa.

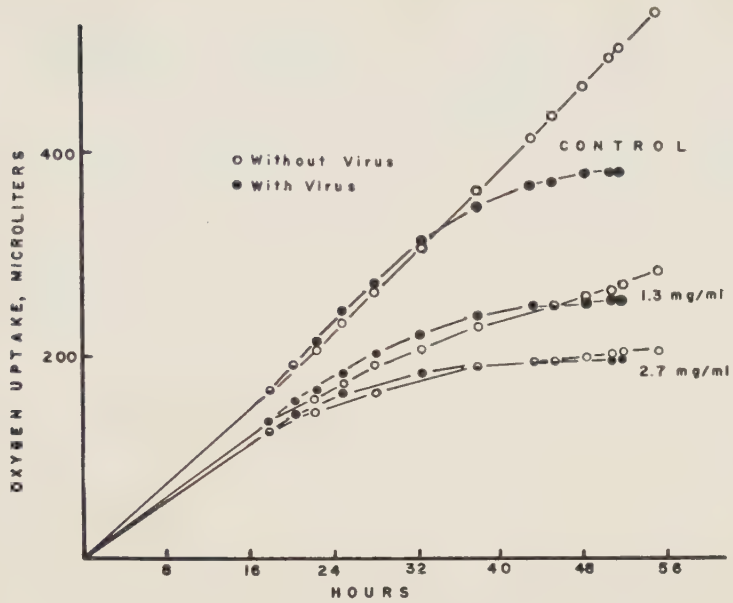

FIG. 4. Effect of ethionine upon respiration of strain HeLa.

these investigators, it is our opinion that the results do not permit definite conclusions.

Much effort is being expended in the search for effective chemotherapeutic and chemoprophylactic agents for virus diseases. Numerous chemical agents have been reported to inhibit virus synthesis in tissue culture. For example, Brown and Ackermann(11) and Brown(12) reported that many chemical agents inhibit poliomyelitis virus replication in tissue culture. It is our experience(13) that the replication of poliomyelitis virus in strain HeLa cellular cultures can not be inhibited by the incorporation of ethionine, benzimidazole, β-2-thienylalanine, 2,6-diaminopurine, pyridine-3-sulfonic acid, DL-desthiobiotin, pantoyl taurine, 3-acetyl pyridine, and desoxypyridoxine HCl in concentrations that do not alter the morphology of HeLa cells. It was shown that at levels of doubtful toxicity, inhibition of virus synthesis was occasionally manifested. These results led to the use of the measurement of oxygen uptake as a more sensitive and objective means for the determination of toxicity. Such non-specific interference with virus synthesis can be recognized readily by the observation of an altered respiratory rate. The existence of a variety of agents, such as 2,4-dinitrophenol, which waste the energy of oxidative metabolism without impairing respiration, would not reduce the value of recognizing those agents which interfere with essential metabolism in a less subtle way.

Summary. DL-ethionine, 2,6-diaminopurine, benzimidazole, and β-2-thienylalanine did not inhibit the synthesis of poliomyelitis virus, Type 1, in strain HeLa cell cultures except at concentrations which were markedly inhibitory to the respiration of the host cell. Cessation of respiration of infected cultures occurred at the same time independent of the concentration of the metabolic analogue employed or of the final concentration of virus. The ethionine-induced inhibition of respiration was only partially reversed by the addition of methionine. A concentration of benzimidazole, 0.25 mg/ml, which was not toxic for HeLa cells, as assessed by microscopic observations, was markedly inhibitory to the respiration of cells in a Warburg flask. The importance of the oxidative energy of respiration for the synthesis of poliomyelitis virus, Type 1, by HeLa cells was demonstrated. Anaerobically, such cells were able to produce less than 0.01% of the amount of virus produced by replicate systems under aerobic conditions. The advantages of a homogene-

ous cell culture over mixed cell cultures for studies of chemical inhibition of virus synthesis are discussed. Cellular uniformity permits precise allocation of the observed effects of a test agent to cells capable of propagating the virus.

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Intestinal Absorption of Vitamin B_{12} in Humans as Studied by Isotope Technic.* (21153)

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The intestinal absorption of vit. B_{12} appears to be variable from one individual to another(1-3) and its underlying principles seem to be contradictory and confusing. Only a small part of vit. B_{12} ingested is absorbed in the intestine(4-6) and the hematopoietic responses of patients with pernicious anemia to oral administration of vit. B_{12} even in association with potent sources of intrinsic factor from human or animal origin are, at their best, lower than those observed after parenteral administration of similar doses of vit. B_{12}(1-3,7-10). On the other hand, fecal excretory studies done with radioactive vit. B_{12} tend to indicate that as much as 90-95% might be absorbed when 0.5-1.0 μg is given orally to normal individuals(11-13).

Recently, we have used surface scintillation measurements of the uptake of radioactive vit. B_{12} by the liver following parenteral and oral administration of this vitamin for the study of B_{12} metabolism(14-16). These investigations have shown that in humans under normal conditions the liver is the target organ for the uptake of radioactive vit. B_{12}, especially after its oral administration and that the scanning technic can be successfully applied to evaluate the intestinal absorption of vit. B_{12}.

Method. The measurements were made with the method described previously(16) in 20 normal individuals or patients with irrevalent disorders, following ingestion of standard doses of Co^{60}-containing vit. B_{12}† to which variable doses of crystalline nonradioactive vit. B_{12} were added. Between the 6th and 10th day after ingestion of radioactive vit. B_{12}, when most of the radioactive material had left the gastrointestinal tract, counts were made with scintillation counter (NaI Thallium 1″ x 1″ crystal) in close approximation with the skin at 1000 volts over 3-4 anterior,

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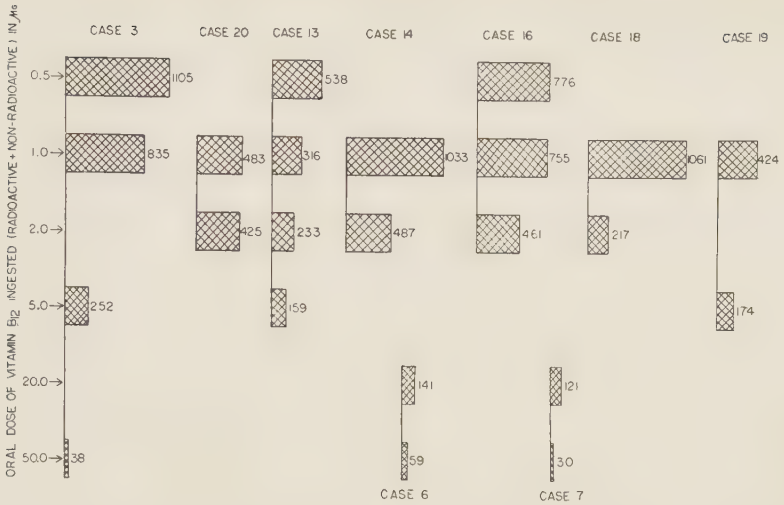

FIG. 1. Averaged hepatic uptake of radioactivity in counts per min. above background in 9 individuals at various oral doses of vit. B_{12} and calculated per 1 microcurie Co^{60}-B_{12} ingested.

antero-lateral, and mid-lateral projections of the liver on 2-4 consecutive days. The counts were measured without amplifier, calculated above background (kept at a low level of about 60 counts per minute), corrected for the efficiency of the scaler with the use of dried Co^{60}-B_{12} standard, and averaged for each day of study. From these multiple averages the mean value of the hepatic uptake was calculated and checked against the mean figure of the averaged abdominal counts which were taken in 6-8 skin projections of the abdominal organs beyond the liver on each day of the study and calculated similarly to the hepatic uptake. These served as a control of the possible scattered radiation emanating from the radioactive material which might still have been retained in the intestines, especially in cases of severe constipation. The mean hepatic counts obtained following ingestion of radioactive B_{12} were then compared with those observed following a similar dose of the vitamin administered parenterally. This allowed one to calculate the parenteral equivalent of the oral dose of vit. B_{12} ingested in the individual tested. Because of the leveling off of the radioactivity counts over the liver during the second week after ingestion of Co^{60}-B_{12}(16) the tests were repeated on the same subjects at intervals of 2-3 weeks with the use of the averaged end counts of the preceding experimental period as the base line for the next experiment.

Results. It soon became evident that there exists an inverse relationship between the radioactivity counts over the liver and the amounts of crystalline vitamin B_{12} added to the radioactive B_{12} ingested. Fig. 1 shows that in the same individual, the uptake of radioactive vit. B_{12} by the liver decreases rapidly on increase of the total dose of vit. B_{12} taken in, when calculated per one μc of radioactive Co^{60}-B_{12} ingested. In view of the law of dilution of radioactive substances in the metabolic pool, this indicates an inverse relationship between the amount of vit. B_{12} ingested and the efficiency of its absorption in the intestine(17). The data in Table I indicate that the oral dose of 0.5 μg B_{12} results in a hepatic uptake of radioactivity equivalent to that observed after intramuscular injection of 90.5 $\pm$ 5.8% of this dose, but that this equivalent rapidly decreases on increase of the intake to amount only to 3.0 $\pm$ 0.7% at the dose of 50 μg B_{12}.

Fig. 2 illustrates the general principle of decreasing efficiency of absorption of vit. B_{12} in the intestine on increase of the dose ingested. The first curve in this figure which has a hyperbolic character, represents the regression of the efficiency of absorption of vit. B_{12} in the intestines with increase of the dose.

TABLE I. Intestinal Absorption of Vitamin B_{12} in Normals at Various Doses of Vitamin B_{12} Ingested.

Dose of radioactive vit. B_{12}, μg	No. of cases studied	Mean hepatic uptake with stand. error in counts/min. above background calculated per 1 microcurie of Co^{60} ingested*	% absorption as compared to parenteral = 100%	Parenteral equivalent of intestinal absorption, μg†
2-10 intramuscularly	4	885 ± 77	100.0 ± 8.7	
.5 orally	3	802 ± 51	90.5 ± 5.8	.45 ± .03
1.0 "	7	784 ± 110	81.5 ± 11.4	.81 ± .11
2.0 "	4	335 ± 72	40.0 ± 8.1	.80 ± .16
5.0 "	3	195 ± 29	22.0 ± 3.3	1.10 ± .16
20.0 "	3	53 ± 13	6.0 ± 1.5	1.20 ± .29
50.0 "	6	26 ± 6	3.0 ± .7	1.50 ± .35

* Each figure represents mean with stand. error averaged from increments in radioactivity uptake over 3-4 skin projections of the liver on several days between 6th and 10th day of experimental period, calculated per 1 microcurie of radioactive Co^{60} contained in dose ingested. Counts were taken with 1" x 1" sodium iodide thallium crystal at 1000 volts without collimator and amplifier, and in close contact with the skin.

† Since at the dose below 20 μg about 6% of vit. B_{12} injected is not retained in the body but excreted in the urine(20) real intestinal absorption figure is at least 6% less than parenteral equivalent listed in this column.

The second curve shows the increments in the absolute amounts of B_{12} absorbed on increase of the dose, which are strikingly small due to the regression of the efficiency of absorption on increase of the intake. Thus, the increment in the amount of vit. B_{12} ingested from 0.5 to 50.0 μg will result in an average increment of vit. B_{12} absorbed from about 0.45 to 1.5 μg, *i.e.*, only about 1 μg. The data have been computed on the basis of a limited number of determinations; individual differences in absorption may be considerable (Table I). It is obvious, however, that the efficiency of absorption of vit. B_{12} in the intestine is best in the physiological range of 0.5-1.0 μg.

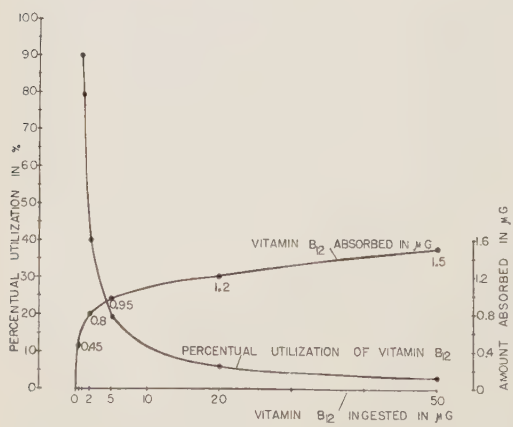

FIG. 2. Average intestinal utilization of vit. B_{12} in normal humans. Curves of the percentual utilization of vit. B_{12} and of the factual amounts of vit. B_{12} absorbed in the intestine were drawn on the basis of data listed in Table I, with exception of the figure corresponding to the dose of 5 μg vit. B_{12} ingested, which was calculated from earlier observations. Because of considerations mentioned in Table I, true values of absorption of vit. B_{12} in intestine are probably by about 6% less than those shown in this figure. Moreover, figures used here should be considered as mean values to which stand. errors listed in Table I should be applied.

Discussion. It appears that the intestinal absorption of vit. B_{12} is controlled by the existence of a partial mucosal block to its absorption which shows much similarity to that existing to the absorption of iron in intestine. There is evidence to show that the partial block to intestinal absorption of vit. B_{12} in normals is not related to the inadequate supply of gastric intrinsic factor of Castle(18,19), and that it cannot be removed in normals by addition of an excess of intrinsic factor containing material(18). This tends to indicate that under normal and pathological conditions it may require for absorption of vit. B_{12}, in addition to gastric intrinsic factor, also an intramural "intestinal B_{12}-acceptor" the role of which in B_{12} metabolism would be similar to that of apoferritin in iron absorption. After B_{12} passes through the mucosal membrane, a process for which the gastric intrinsic factor seems to be necessary(20), the vit. B_{12} would become bound to this B_{12}-acceptor. With in-

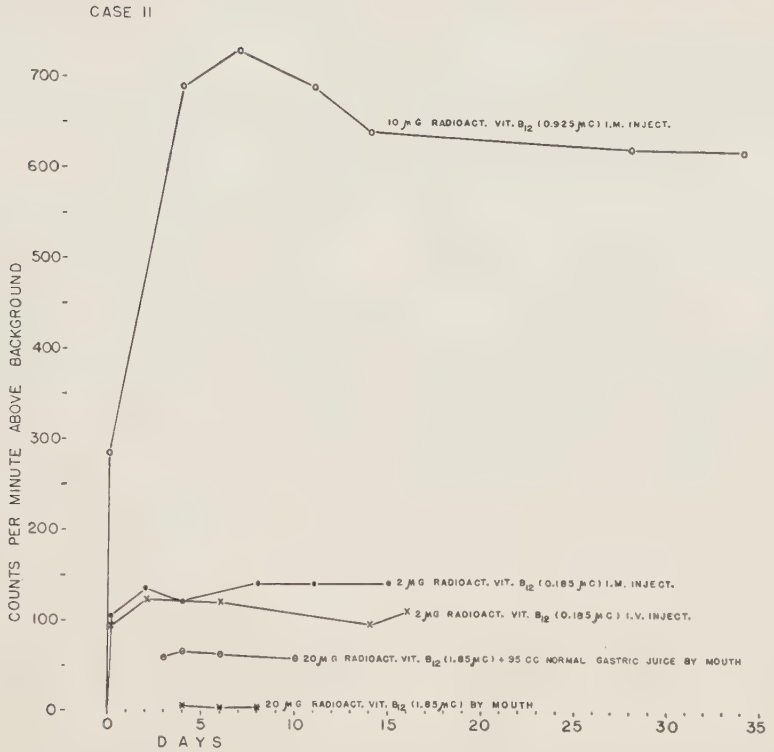

FIG. 3. Uptake of radioactive vit. B_{12} by liver in a patient with pernicious anemia in partial relapse following oral administration of this vitamin alone and with normal gastric juice, as well as after intramuscular and intravenous administration. The parenteral dose was 2 respectively 10 times smaller than the oral one which was 20 μg radioactive vit. B_{12} containing 1.85 microcuries of Co^{60}.

creasing saturation of the B_{12}-acceptor in the intestinal wall the absorption of vit. B_{12} in the intestine would be braked, which might explain the regression of efficiency of absorption of vit. B_{12} on increase of the dose. The gradual release from the intestine of B_{12} bound to this hypothetic intestinal B_{12} acceptor may also explain why it takes up to 7 days for the hepatic uptake of the orally administered dose of radioactive B_{12} to come to a peak(14-16). There is evidence that after intestinal absorption B_{12} becomes bound to one of the proteins in the serum(3,21,22), which, by analogy with the serum transferrin we might call "B_{12}-transferrin" and the function of which would consist in carrying the bound vit. B_{12} in blood. Ultimately, vit. B_{12} becomes anchored in the liver(14,15) and the hematopoietic tissues.

The partial mucosal block to intestinal absorption of vit. B_{12} changes to a complete or almost complete block in sprue and in pernicious anemia, as evidenced by no or a negligible hepatic uptake of orally administered vit. B_{12} in these diseases(14-16). In sprue, the block cannot be corrected by the addition of gastric intrinsic factor(14-16), because the defect in this disease depends on a generalized and inherent defect in the absorption mechanism of the intestinal wall. In pernicious anemia, the block to absorption of vit. B_{12} depends largely on the absence of Castle's gastric intrinsic factor and can be converted into a partial block, similar to that existing in normals, by addition to vit. B_{12} of normal human gastric juice(1,2,7,10), intrinsic factor concentrate from human or hog stomach (7-9), or by lavishly increasing the intake of vit. B_{12} alone, which through mass effect overcomes the block and results in absorption of some small fraction of the ingested dose(2,4). However, the principle of regressing efficiency

of absorption with increase of the intake still will hold under these circumstances (Fig. 3). In this patient with pernicious anemia the hepatic uptake of radioactivity was zero following the ingestion of an oral dose of 20 μg Co^{60}-B_{12} alone, but it became equivalent to about 5% of the similar parenteral dose when it was given together with a potent source of intrinsic factor. This is the usual range of efficiency of intestinal absorption of vit. B_{12} at the intake of 20 μg in normals (Table I).

The principle of regressing utilization of vit. B_{12} in the intestine permits a better understanding of difficulties encountered in the oral treatment of pernicious anemia with vit. B_{12}, and the apparent "unpredictability" of hematopoietic responses under these circumstances.

Summary. The scintillation measurements of the hepatic uptake of Co^{60}-B_{12} following its oral and parenteral administration to 20 normal humans, indicate that the efficiency of intestinal absorption of vit. B_{12} decreases sharply on increase of the intake. The peak of the absorption curve of vit. B_{12} was found at the oral dose of 0.5 μg, at which the hepatic uptake was found equivalent to 90.5 $\pm$ 5.8% of that observed following intramuscular injection of a similar dose of this vitamin. With the increase of the dose, a progressive decline in absorption followed a hyperbolic regression curve, so that at the oral dose of 50 μg B_{12} the hepatic uptake was equivalent to only 3.0 $\pm$ 0.7% of that found after intramuscular injection of a similar dose of this vitamin. The data obtained indicate that the increment in the oral dose of vit. B_{12} from 0.5 to 50.0 μg, results apparently in an increase of the amount absorbed of only 1.0 μg. It is suggested that in addition to Castle's gastric intrinsic factor, an intramural "intestinal B_{12}-acceptor" exists, which may be responsible for the partial mucosal block to the absorption of vit. B_{12} in the intestine of normal humans. The role of this hypothetic acceptor in the absorption of vit. B_{12} might be analogous to that of apoferritin in intestinal absorption of iron.

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Certain Immunological Relationships between Human Erythrocytes and Selected Mouse Cells. (21154)

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In the course of an immunological study of cells obtained from the mouse, it was observed that antiserums prepared against the mouse cells were capable of agglutinating human erythrocytes. Landsteiner(1) had previously observed the agglutination of mouse erythrocytes by antihuman B serum and later Gorer(2) was able to differentiate at least 2 antigenic types of mouse corpuscles by means of normal anti-human A serum. Because our anti-mouse cell serums were capable of agglutinating A, B, and O human erythrocytes, it seemed of interest to investigate the reactions of these anti-mouse cell serums with human red blood cells.

Materials and Methods. Erythrocytes of humans and of C3H and Webster mice were obtained from citrated whole blood. These cells were washed 3 times with veronal buffered saline* and the contaminating leucocytes were removed, in so far as possible, by pipetting, following each centrifugation.

Leucocytes were obtained from Webster mice following intraperitoneal injection of 5 ml of a 1% suspension of Aleuronat in Locke's solution. After a period of 27 hours, the mice were sacrificed and 5 ml of saline was injected intraperitoneally. The abdomen was massaged, the peritoneal cavity opened by a mid-line incision, and the suspended leucocytes were removed with a capillary pipette. The cells so obtained were placed in a tube containing sufficient 10% sodium citrate to give a final concentration of 3%. The cell suspension was mixed and allowed to stand at 25°C for 30 minutes. The cells which remained suspended were removed and washed 3 times in veronal buffered saline. After each centrifugation during the washing procedure the contaminating erythrocytes, which are aggregated by centrifugation, were removed by a capillary pipette.

The L-strain of mouse fibroblasts was isolated by Earle(3) from the C3H mouse and later was developed into a pure cell strain(4). These cells were grown in 3-ounce prescription bottles in a medium containing 50% Hanks's balanced salt solution, 10% chick embryo extract, and 40% horse serum or human ascitic fluid. The cells were harvested by shaking them free from the glass surface. After being centrifuged and resuspended in veronal buffered saline the cells were allowed to stand at 25°C for 30 minutes; the suspended cells were removed and washed 3 times in veronal buffered saline.

All the above cells were used in serological tests on the same day they were obtained.

To standardize the erythrocyte suspensions the cells in a 1 ml aliquot were lysed by the addition of distilled water to a volume of 10 ml following which readings were made on the lysed suspension in the Klett-Summerson colorimeter. Direct turbidimetric readings were made on the L-strain cells and the leucocytes. Calibration curves were prepared for each of the cell types by plotting %-cell concentration (from packed cell volume) against Klett readings. Fairly reproducible cell suspensions could be prepared from these curves.

Antiserums for the 3 types of mouse cells were prepared in rabbits by giving a series of 3 weekly injections of 1 ml of a 5 to 10% suspension of the cells for a period of 3 weeks; intraperitoneal, subcutaneous, and intravenous injections were given alternately. In certain instances 1 to 3 additional booster injections were given. The animals were bled 10 days after the final injection.

Anti-human A and B serums were obtained

* 85.0 g NaCl, 5.75 g 5,5-diethyl barbituric acid, 3.75 g sodium 5,5-diethyl barbiturate. Dissolve the acid in 500 ml hot water, add to the solution of the other components, cool and make up to 200 ml with water. Each day dilute accurately 1 part up to 5 with water; the pH of the diluted buffer should be 7.3 to 7.4.

from individuals of type A and type B blood groups.

Agglutination tests were carried out in 10 x 75 mm tubes in which 0.1 ml of the antigen suspension (0.5% for erythrocytes and 0.75% for the leucocytes and the L-strain cells) was added to 0.2 ml of the serum dilution. The tests were incubated at 37°C for 1 hour, then centrifuged at 1000 rpm for 2 minutes. The tubes then were shaken vigorously and the agglutination was read with the aid of a microscope lamp. The tests were checked again after standing at 25°C for a period of 2 to 3 hours. There was usually no change in titer during this second incubation period; however, there was a tendency for the weak reactions to become stronger. The highest dilution of the serum showing discrete clumping of the cells, as compared to the control, was taken as the end point.

Antiserums were absorbed 3 times, with a 5% absorbing dose of cells, for 30 minutes each time, at 25°C. Such treatment was sufficient to remove all the antibody from a 1:10 dilution of the homologous antiserum.

The absorption procedure was as follows: A sufficient amount of washed cells to give a 5% cell concentration in a volume of 1.5 ml was added to a McNaught sedimentation tube. This tube was centrifuged for 15 minutes at 2300 rpm in a Clay-Adams Safety-Head centrifuge and the supernate was carefully removed and discarded. One and one-half ml of an appropriate dilution of the antiserum to be absorbed was added and the cells were resuspended. After a 30-minute incubation period at 25°C the suspension was centrifuged and the antiserum was removed and absorbed twice more in a similar manner. It was found that a volume correction for the saline contained in the absorbing mass was not necessary.

TABLE I. Agglutinating Titers of Anti-Mouse Cell Serums with Human RBC's.

Antiserum	Antigens A	B	0 pos.	0 neg.
Webster-RBC	32	32	8	8
C3H-RBC	8	32	8	4
Webster-WBC	16	16	8	4
L-strain	4	8	2	0
Nor. S.	0	0	0	0

TABLE II. Agglutinating Titers of Anti-Mouse Cell Serums with Human RBC's after Absorption with Group A Rh Pos. Human RBC's.

Antiserum	Antigens A	B	0 pos.	0 neg.
Webster-RBC	0/32*	32/32	0/8	0/8
C3H-RBC	0/8	32/32	0/4	0/2
Webster-WBC	0/16	16/16	0/8	0/4
L-strain	0/2	8/8	0/0	0/0

* Numerator = Titer of absorbed serum. Denominator = Titer of unabsorbed serum.

Unabsorbed serums were tested at the same time as the absorbed serums to eliminate the variation in titer due to differences in serological reactivity of the cell suspensions.

Experimental. Table I gives the results of agglutination of human erythrocytes with anti-mouse cell serums. It may be noted that group A,B,O Rh positive and O Rh negative human erythrocytes were agglutinated by each of the antiserums (with the exception of L-strain antiserum and O Rh negative cells), but not to the same titer. Serum from 3 nonimmunized rabbits did not agglutinate the human cells tested.

TABLE III. Agglutinating Titers of Anti-Mouse Cell Serums with Human RBC's after Absorption with Group B Rh Pos. Human RBC's.

Antiserum	Antigens A	B	0 pos.	0 neg.
Webster-RBC	32/32*	0/32	0/8	0/8
C3H-RBC	8/8	0/32	0/8	0/4
Webster-WBC	16/16	0/16	0/8	0/4
L-strain	2/2	0/8	0/0	0/0

* Numerator = Titer of absorbed serum. Denominator = Titer of unabsorbed serum.

It was of interest to determine whether more than one antibody was responsible for the agglutination of the human erythrocytes. This was done by absorption of the anti-mouse cell serums with human erythrocytes of the different blood groups. The results are given in Tables II to IV.

Table II indicates that anti-mouse cell serums absorbed with group A human erythrocytes failed to react with Group A, group O Rh positive, and group O Rh negative erythrocytes, but were still able to react

TABLE IV. Agglutinating Titers of Anti-Mouse Cell Serums with Human RBC's after Absorption with Group B Rh Neg. Human RBC's.

Antiserum	Antigens A	B	0 pos.	0 neg.
Webster-RBC	32/32*	0/32	0/8	0/8
C3H-RBC	8/8	0/32	0/8	0/2
Webster-WBC	16/16	0/16	0/8	0/8
L-strain	2/2	0/8	0/0	0/0

* Numerator = Titer of absorbed serum. Denominator = Titer of unabsorbed serum.

with group B cells. Similarly (Table III), absorption with group B cells removed antibodies reactive with group B and group O cells leaving antibodies reactive with group A cells. Table IV indicates that absorption with Rh negative cells removed antibodies against group O Rh positive as well as group O Rh negative cells.

To substantiate the relationships between the mouse cells and the human erythrocytes, cross-reaction tests between the mouse cells and isoantibodies were made. The results (Table V) indicate that each of the 4 types of mouse cells were agglutinated by both the anti-A and anti-B serums.

Discussion. It was found that antiserums prepared against selected mouse cells were capable of agglutinating human erythrocytes of the various blood groups indicating an antigenic relationship between human erythrocytes and the mouse cells.

TABLE V. Agglutination Titers of Mouse Cells with Human Iso-Anti-A and Iso-Anti-B Serums.

Anti-serum	Antigens Webster RBC	C3H RBC	Webster WBC	L-strain	Human RBC Gr. A	Gr. B
Iso-A	8	4	4	16	32	—
Iso-B	16	4	8	8	—	8

From the results of absorption with human erythrocytes, it was concluded that the anti-mouse cell serums contain antibodies specific for the group A and group B substances. Further evidence of the presence of both of the group substances in the individual mouse cell types was obtained from the aggutination of the mouse cells by either A or B isoantibody.

There was evidence of a further relationship between human erythrocytes and mouse cells independent of group A, group B, or Rh antigens. This relationship was indicated when antibodies against the group O cells were absorbed by either A or B cells regardless of whether they were Rh positive or negative.

Summary. 1. It was found that erythrocytes and leucocytes from Webster mice and erythrocytes and L-strain fibroblasts from C3H mice contain antigens related to A and B human blood group substances. 2. There was an indication of the presence of further immunological relationships between human erythrocytes and mouse cells independent of group A and B substances and in the Rh factor.

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Observations on Pharmacology of the Anticholinesterases Sarin and Tabun. (21155)

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The following account summarizes observations made in late 1947 and early 1948 on the pharmacology of sarin and tabun, potent anticholinesterase nerve gases, which, according to Holmstedt(1), were first synthesized by Schrader and were once considered by German military authorities as chemical warfare agents.* Wood, and Grob and Harvey have discussed certain aspects of the action of such anticholinesterase compounds elsewhere(2,3). The structure of sarin and tabun is compared in Fig. 1 with di-isopropylfluorophosphate

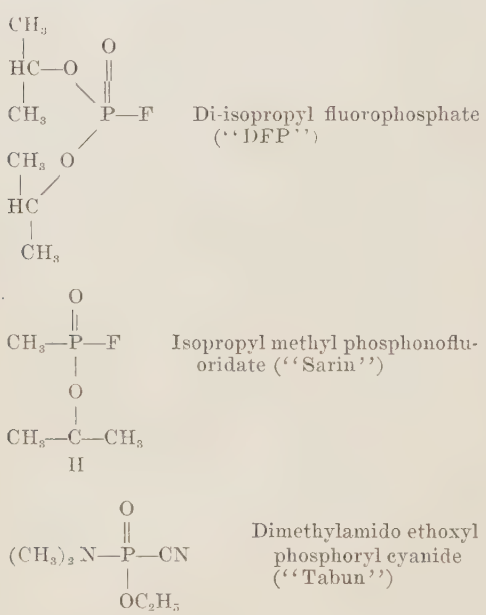

FIG. 1.

(DFP), another well known anticholinesterase compound. The compounds were dissolved in propylene glycol, and were administered in this form intravenously in all experiments summarized in this report.†‡

The following experiments were performed to define the mechanism of toxic action and lethal outcome in cats and dogs.

1. *Signs of poisoning.* After intravenous administration of 20 μg of sarin per kilo§ to cats and dogs, the animals became apprehensive and showed signs of respiratory distress in 15 to 30 seconds. The respiratory rate became somewhat accelerated, while the respiration became deep and apparently labored. Within the next 15 to 30 seconds, cyanosis began to appear, and approximately 30 seconds later, cyanosis became extreme. In experiments uncomplicated by anesthesia,‖ increasing signs of excitement occurred simultaneously and, particularly in cats, severe convulsions supervened after a period of marked unrest, variable in duration, interspersed with intervals of aimless, fearful running about. Miosis under these conditions was not seen, although under anesthesia miosis was seen before cyanosis appeared. Generally, intense salivation became evident at the time that severe respiratory difficulty (gasping) appeared. In cats, pilomotor activity was frequently seen.¶

* Earlier publication was not possible. Since completion of the work described here, Holmstedt(1), and De Candole(4) have published reports on tabun and sarin respectively, which include many of the observations presented here.

† The compounds were prepared in other laboratories of the Chemical Corps.

‡ In general, the effects of sarin and tabun were qualitatively similar in the dog and cat, sarin being from 5 to 10 times more potent; hence, only the effects of sarin are described in this report.

§ All doses here reported are per kilo of body weight.

‖ Since the central nervous system (CNS) effects of DFP may be suppressed by anesthesia, some observations on symptoms and signs of poisoning were required in unanesthetized animals to determine whether convulsant CNS effects of sarin and tabun exist to an extent presenting a problem in treatment of poisoning.

¶ In the monkey, excitement and convulsions were so fleeting as to appear negligible, and the animals passed quickly into a state of unconsciousness, flaccidity, and cessation of respiration. (Unpublished, recent observations, Loomis and Krop)

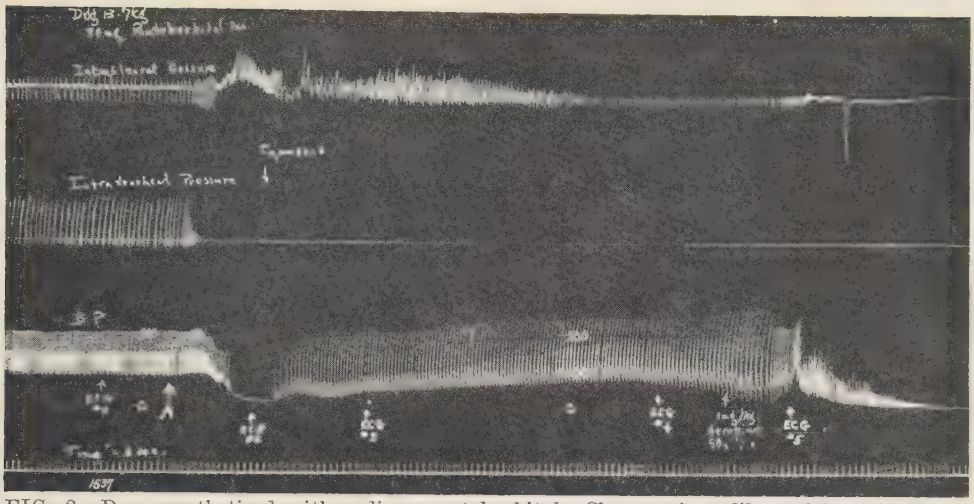

FIG. 2. Dog anesthetized with sodium pentobarbital. Changes in caliber of bronchial tree measured by simultaneous intrapleural and intratracheal pressure by means of rubber membrane tambours. Blood pressure recorded by means of a rubber membrane calibrated manometer.

Defecation and urination occurred. Death commonly followed in 5 minutes or less; often, animals surviving the first 5 minutes of poisoning ultimately recovered, showing general weakness for long periods. Prior to death, and starting with the excitement and convulsive phenomena, skeletal muscular twitchings appeared. These continued for some minutes after the convulsive seizures, and after respiratory and cardiac activity ceased. Death appeared to be primarily asphyxial in some instances and primarily cardiovascular in others; and in some cases, failure of both seemed to coincide.

These observations suggested that an obstruction of the respiratory tract plays an important part early in the picture of poisoning by these compounds. Indeed, at a time when the animals were capable of executing vigorous respiratory movements and the heart beat was palpable, cyanosis was observed to be extreme. Since phonation or stridor were absent, severe respiratory tract obstruction below the larynx was suspected. Considering the rapid development of the respiratory embarrassment, the hypersecretion of fluid in the respiratory tract did not appear to be the dominant or decisive factor in the presumed respiratory obstruction despite the eventual appearance of intense salivation in consequence of the known "para-sympathomimetic" properties of anticholinesterase agents. The more likely explanation for early respiratory tract obstruction resulting in cyanosis appeared to lie in the response of the bronchial, and perhaps other intrapulmonary, smooth musculature after intravenous administration of these compounds. Considering these facts, the following experiments were performed.

2. *Action of sarin on respiration.* Intrapleural and intratracheal pressure changes below the larynx were recorded in anesthetized (pentobarbital sodium) cats and dogs by means of tambours of requisite sensitivity by the method of Jackson. Simultaneously, a continuous record of carotid arterial blood pressure was made and electrocardiograms were taken at frequent intervals (Fig. 2). Fifteen to 20 seconds after intravenous injection of 10 μg of sarin, respiration became moderately increased in depth and rate. Intrapleural pressure between respiratory cycles tended to approach positive (atmospheric) pressure, while inspiratory (negative) and expiratory (positive) became greater, reflecting greater muscular effort applied to the thorax in intake and expulsion of air. During the succeeding 15 seconds or so, the intratracheal pressure changes in each respiratory cycle—a measure of the tidal volume—declined very rapidly, in 30 seconds became absent, and simultaneously cyanosis became severe. This

"bronchoconstriction" was seen most consistently and completely in the dog. Vigorous inspiratory and expiratory efforts (intrapleural pressure changes) continued for several minutes, or until death or reversal of effects by an antidote.** Evidence for "bronchoconstriction" was also obtained in anesthetized, curarized dogs under positive pressure artificial respiration, *viz.*, a progressive rise in intratracheal and a progressive fall in intrapleural pressures during each inflation cycle in consequence of sarin action.

3. *Action of sarin on the cardiovascular system.* Thirty to 60 seconds after intravenous injection of 10 to 20 μg of sarin per kilo, the arterial blood pressure record, taken with a calibrated rubber membrane manometer, showed a sharp, moderate rise in systolic pressure followed by a marked fall accompanied by slowing of the heart. Cardiac arrest often occurred, requiring ½ to one minute for "escape" of the heart to resume a rate of 20 to 30 beats per minute until death of the animal or relief of the bradycardia by an antidote. Despite this bradycardia, the mean arterial blood pressure often tended to rise above pre-poisoning levels during this period prior to death. The onset of the severe bradycardia was very commonly associated with the decline in the "tidal volume," but often bradycardia did not appear until the reduction in tidal volume was well advanced.

The electrocardiogram showed an initial slowing with normal rhythm, followed by progressive prolongation of the P-R interval and ultimately complete block, with disappearance of the P wave, when the ventricular rate became 20 to 30 per minute; the T wave showed inconstant changes in direction and degree, and the QRS complex became lengthened with frequent "notching" of the R spike. The electrocardiogram often continued at a low rate after the arterial pressure and respiration failed.

4. *Duodenal activity.* Activity of the duodenum of anesthetized cats and dogs was observed utilizing the hydraulic system of Krop and Loomis(5). Duodenal activity is extremely susceptible to the influence of these compounds, responding with increased tonus and rhythmicity to doses producing no discernible effects on respiration or circulation. Doses producing respiratory and circulatory effects resulted in intense spastic contraction of the duodenum generally within one to 2 minutes.

5. *Effects of atropine.* Control of the "muscarinic" and of certain central nervous system effects of di-isopropylfluorophosphate by atropine has been reported elsewhere(6-8). One tenth to one mg of atropine sulfate intravenously given prophylactically or after administration of sarin in the amounts here reported prevented or abolished the effects of sarin described in the foregoing. Particularly striking was the abolition of the excitement and convulsions in the cat. Although the excitatory effects on the nervous system, and the effects on respiratory obstruction and circulation were controlled by atropine to a life-saving degree when given after doses of sarin described here, death from larger doses of sarin was not prevented presumably due either to a paralytic action of sarin on the central nervous system or peripheral neuromuscular block of the respiratory apparatus (6). Occasionally, atropine precipitated fatal ventricular fibrillation when given late in the course of poisoning.

Discussion. Sarin and tabun exert marked "muscarinic" and "nicotinic" effects in cats and dogs in very small doses, presumably by virtue of inhibition of cholinesterases(1). The observations reported here suggest that the principal mechanisms of lethal action of these phosphate esters given intravenously to the cat and dog center on the lung, the circulation and the central nervous system. Any of these 3 effects alone appeared sufficient to result in a lethal outcome. The ventilatory resistance and the attendant cyanosis which develops early in poisoning appears to be chiefly due to contraction of the smooth musculature of the lower respiratory tract, *i.e.*, "bronchoconstriction," including possibly other intrapulmonary smooth musculature. These are simi-

** Recently, similar effects have been observed by us in unanesthetized dogs by means of a body plethysmograph (for respiratory tidal volume) and an esophageal balloon (to reflect intrapleural pressure changes), the latter being a technic applicable to similar problems in man.

lar to the effects of choline esters and other parasympathomimetic drugs described by others(9). The pattern of intrapleural pressure changes during the respiratory cycle indicated that in some instances, early in the course of poisoning, air may be trapped in the lung producing an "asthmatic" condition. That "bronchoconstriction" should occur following inhalation of high concentrations of sarin and tabun vapor seems certain, since this route of administration closely resembles intravenous administration in many respects, and provides for contact of pulmonary smooth musculature with high concentrations. Hypersecretion of fluid in the lower respiratory tract, though undoubtedly occurring, can hardly account for the great rapidity with which ventilatory resistance develops and with which it is relieved by atropine under the conditions of our experiments. The transient cardiac arrest and subsequent bradycardia could in themselves account for cyanosis in the early stages of poisoning, since the blood might not be circulated through the lung at a rate adequate to maintain a degree of hemoglobin oxygenation required to prevent cyanosis and tissue hypoxia. Convulsive seizures produce fatigue and by themselves prevent adequate pulmonary ventilation. Although the successful treatment of sarin and tabun poisoning rests fundamentally on the prevention of anoxia, the simultaneous control of all 3 effects described here must be achieved. For example, barbiturates prevent the excitatory and convulsive actions effectively, but "bronchoconstriction" is unaffected and death may be asphyxial nonetheless; similarly, if the cardiovascular effects could be counteracted without relieving ventilatory resistance, asphyxia would nevertheless also develop.

Artificial respiration by conventional methods (manual) may not be relied upon to bring about the recovery of an animal severely poisoned by these agents, even if depressed blood circulation could be disregarded, so long as severe "bronchoconstriction" persists. With a positive pressure method adequate for the task, there can be no assurance that patches of "emphysema" will not be blown into some portions of the lung leaving patches of "atelectasis" in others; further, secretions may be blown into the lung. Moreover, artificial respiration alone, no matter how effectively the alveoli may be ventilated thereby, will not wholly relieve the marked vascular stasis consequent upon the bradycardia. The aim in treatment should therefore be to assure an airway patent functionally, to assure that the circulation is not impeded, and to prevent the marked stimulation (convulsions and attendant interference with artificial respiration) and subsequent depression of the central nervous system. These requirements appear to be met within limits by readily tolerable doses of atropine, thus providing physiological conditions optimal for treatment of poisoning from these and other compounds similar in action such as the insecticide tetraethylpyrophosphate.

Summary. The phosphate esters sarin and tabun, potent anticholinesterase compounds, exert profound effects on the respiration, circulation, central nervous system and gastrointestinal tract of experimental animals.

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Tetrazolium Stains for Diphosphopyridine Nucleotide (DPN) Diaphorase and Triphosphopyridine Nucleotide (TPN) Diaphorase in Animal Tissue.* (21156)

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Seligman and coworkers(1,2) have recently described a method of staining for the succinic dehydrogenase system using tetrazolium salts as indicators. Their method has proved satisfactory in the hands of several other investigators(3,4). For the past 2 years, we have been attempting to develop technics for the histochemical demonstration of other oxidative enzymes. Methods employing tetrazolium salts have now been perfected for the demonstration of 2 enzymes, diphosphopyridine nucleotide (DPN) diaphorase and triphosphopyridine nucleotide (TPN) diaphorase.

In our original experiments, frozen sections of animal tissues (mainly rat kidney) were incubated in solutions containing tetrazolium salts together with factors which should permit the activity of individual tissue dehydrogenases. Excellent staining was observed in many instances. For example, when sections were incubated in a medium containing malate as substrate with cofactors and the coenzyme (DPN) essential for malic dehydrogenase activity, distinctive staining patterns were observed in the sections. Omission of any one of these components prevented staining. The localization of formazan in the tissue was highly reproducible and was different from that observed with the succinic dehydrogenase system. However, when the composition of the incubating medium was changed so as to permit the action of lactic dehydrogenase, which is also a DPN-linked enzyme, the staining pattern was identical to that observed with the malic system. Again, when sections were incubated in media containing alcohol as substrate and soluble yeast alcohol dehydrogenase (crystalline) as the only active DPN-linked dehydrogenase in the medium or tissue, the staining patterns were identical to those obtained with the malate and lactate systems.

It soon became clear that the action of the dehydrogenases was not directly responsible for the staining and probably served only to produce a common factor essential to the activity of some other enzyme in the tissue. This theory gained support when identical staining patterns were obtained on incubating sections in media that contained no dehydrogenase substrate but only reduced DPN.

Similarly, with TPN-linked system, it was found that each of 3 different dehydrogenases (isocitric, glucose-6-phosphate, and Ochoa's "malic enzyme") gave identical staining patterns which were highly reproducible but differed from those obtained with the DPN systems or the succinic dehydrogenase system. Here too, it appeared that the role of the dehydrogenases might be merely to produce a substrate for some other enzyme.

The enzymes responsible for these 2 distinct staining patterns have been tentatively identified as DPN diaphorase and TPN diaphorase. This conclusion is in agreement with that of Brodie and Gots(5,6) who demonstrated that a flavoprotein enzyme is essential for reduction of tetrazolium salts by several dehydrogenase systems.

Frozen sections of tissue prepared in the routine manner contain insufficient amounts of the dehydrogenase substrates and the coenzymes DPN or TPN to allow significant dehydrogenase activity but do retain the diaphorases and effective amounts of many dehydrogenase apoenzymes. Therefore, staining specific for DPN diaphorase may be obtained

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† Scholar in Cancer Research of the American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

by incubating frozen sections in a medium containing DPN and tetrazolium together with substrates and cofactors for one or more DPN-linked dehydrogenases. In like fashion TPN diaphorase is selectively stained by supplying TPN and the factors necessary for the action of a TPN-linked dehydrogenase. Increased efficiency of tetrazolium reduction may be obtained by permitting 2 dehydrogenases to react simultaneously with the same coenzyme. In this way, the DPN or TPN is reduced at a more rapid rate to furnish a richer substrate for the action of the diaphorase.

The following incubation medium has yielded reproducible and satisfactory staining for DPN diaphorase in frozen sections of rat kidney and other tissues: Solution of crystalline alcohol dehydrogenase (Mann) (ca. 1.5 mg per ml)—0.1 ml, ethyl alcohol (1.09M)—0.2 ml, DPN (5 mg per ml)—0.2 ml, Na L-malate (0.5M)—0.3 ml, Na L-glutamate (0.5M)—0.5 ml, semicarbazide (0.1M)—0.2 ml, blue tetrazolium (1 mg per ml)—0.7 ml, and phosphate buffer (0.1M, pH 7.4)—0.8 ml. All solutions are adjusted to pH 7.4 before use. The alcohol is supplied as substrate for the added alcohol dehydrogenase. The malate serves as substrate for the endogenous malic dehydrogenase. Oxalacetate, the product of malate oxidation, will "poison" the enzyme system if allowed to accumulate. Glutamate added to the medium removes oxalacetate by converting it to aspartate through the action of transaminase. The semicarbazide removes the acetaldehyde formed by oxidation of the ethanol as well as reacting with oxalacetate.

This system is planned so as to permit both endogenous (malic) and exogenous (alcohol) dehydrogenases to contribute to the supply of reduced DPN. Although neotetrazolium can be used in place of blue tetrazolium, we have found blue tetrazolium more satisfactory. The medium usually remains colorless if the alcohol dehydrogenase solution is made up not more than a few weeks before it is used.

For staining for TPN diaphorase, the following medium has proved satisfactory: A mixture of Na L-malate and Na citrate (each 0.5M)—0.3 ml, TPN (2 mg per ml)—0.1 ml, $MnCl_2$ (0.005M)—0.3 ml, cysteine hydrochloride (0.1M)—0.2 ml, veronal buffer

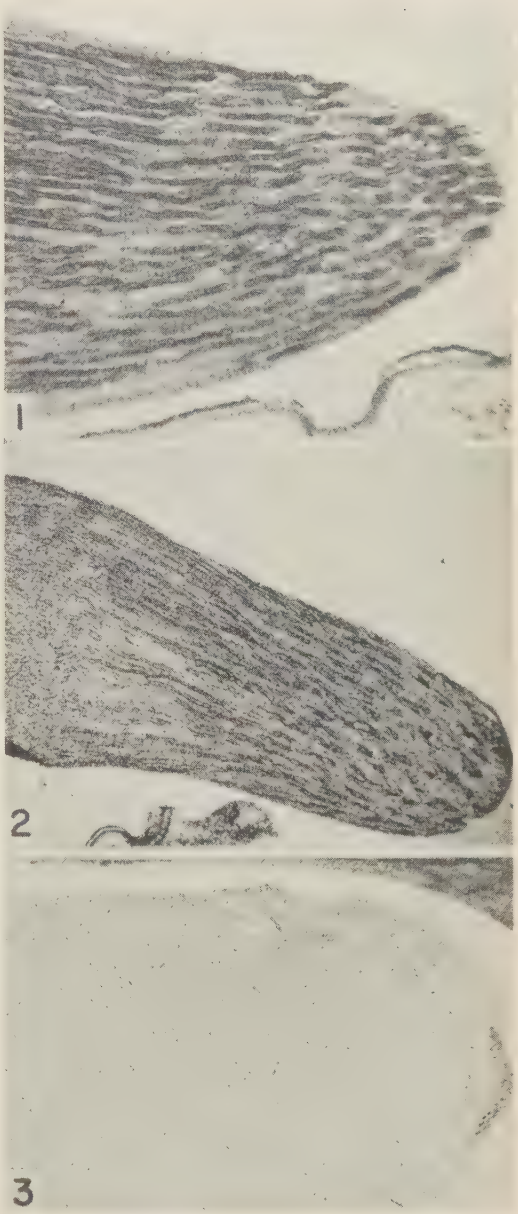

FIG. 1-3. Papilla of the rat kidney stained for DPN diaphorase, TPN diaphorase, and succinic dehydrogenase system.

FIG. 1. DPN diaphorase. Collecting tubules stain intensely throughout their length. Moderate staining of epithelium of renal pelvis.

FIG. 2. TPN diaphorase. Note increasing intensity of staining of collecting tubules as tip of papilla is approached.

FIG. 3. Succinic dehydrogenase system. Virtually no staining of the rat kidney papilla.

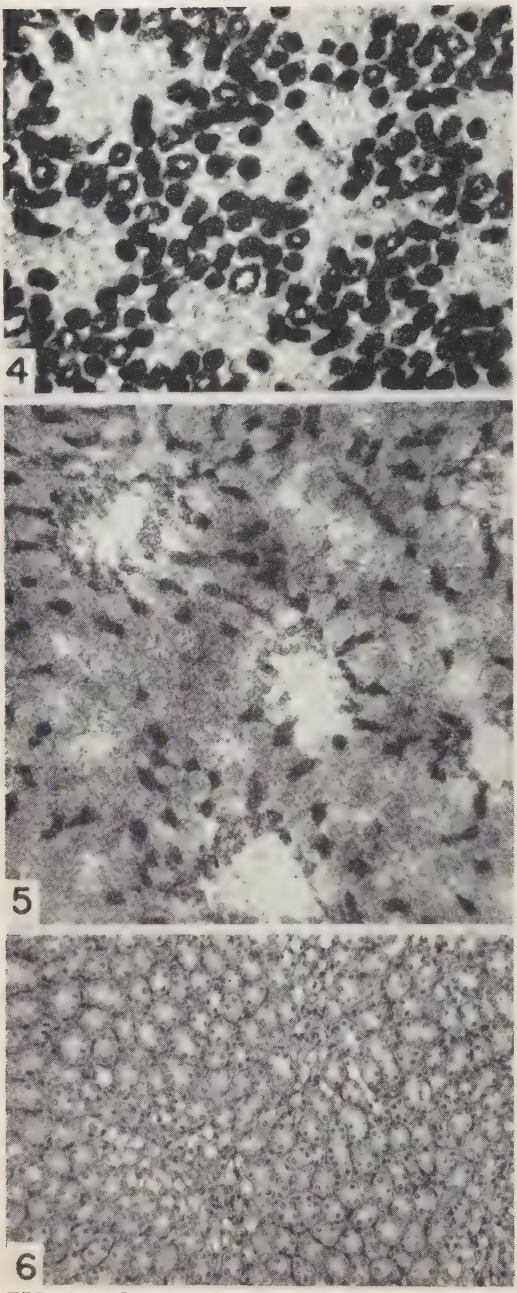

FIG. 4-6. Cross-sections through outer medullary zone of rat kidney, stained for DPN and TPN diaphorase, with a hematoxylin and eosin stained paraffin section for comparison.

FIG. 4. DPN diaphorase. Two types of tubules are readily identified and stain deeply. Predominant tubules are the ascending thick limbs of Henle with small or indistinguishable lumens. Collecting tubules are larger and their lumens wider. Delicate staining of endothelium of the vascular bundles *(vasa recta)* is also seen.

FIG. 5. TPN diaphorase. Larger, pale-staining tubules (pink in the original preparation) are ascending thick limbs of Henle. Intensely stained small-caliber tubules are thin limbs of Henle (deep blue in the original preparation). Relatively clear areas are vascular bundles *(vasa recta)*.

FIG. 6. Hematoxylin and eosin stain, same magnification as Fig. 5. Thick limbs of Henle predominate. Thin limbs of Henle are difficult to identify or to differentiate from blood vessels.

0.005M, pH 7.4)—0.9 ml, methyl cellulose (15 cps. 3%)—0.5 ml, and blue tetrazolium (1 mg per ml)—0.7 ml.

The malate is supplied as substrate for the TPN-linked "malic enzyme" of Ochoa (malic decarboxylase); the citrate is converted to isocitrate by the active tissue aconitase and the isocitrate then serves as substrate for the tissue isocitric dehydrogenase. Mn^{++} is an essential activator for the decarboxylation of malate and also oxalsuccinate, the product of isocitrate oxidation. Cysteine or a suitable substitute is essential in the system and no reduction of tetrazolium occurs in its absence. In the absence of substrate, cysteine produces no staining or at most a very faint pink. Fe^{++} cannot replace cysteine. Thioglycollate but not glutathione can effectively substitute for cysteine but a higher concentration is required. The cysteine must be freshly prepared and neutralized. Veronal buffer was used in place of phosphate buffer to prevent any possible inhibition of aconitase by phosphate. The methyl cellulose is not essential but its presence gives better stained preparations. With all TPN systems, the medium tends to become colored due probably to leaching out of the TPN diaphorase from the tissue into the incubating solution. By adding the viscous methylated celluloses, coloration of the medium is much reduced, and staining of the tissue is considerably enhanced. Different methyl celluloses (Methocel, Dow Chemical Co.) or carboxymethyl celluloses (Hercules Powder Co.) give somewhat different degrees of improvement of the staining but all enhance it to at least some degree.

In the staining for both diaphorases, frozen sections are floated in cold phosphate buffer (0.1M, pH 7.4) and then transferred to 50 ml glass stoppered Erlenmeyer flasks containing the cold incubating media. The flasks

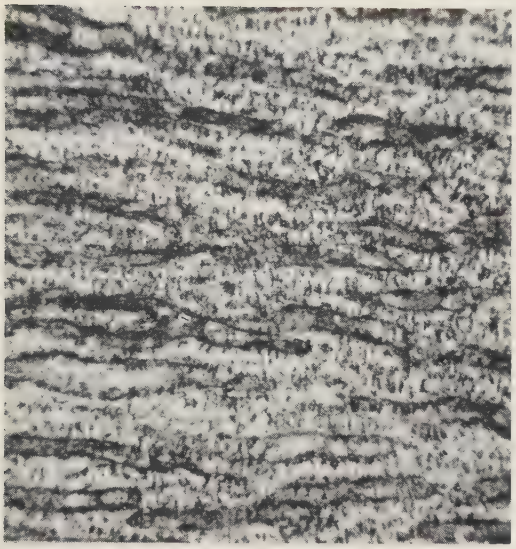

FIG. 7. TPN diaphorase stain of distal third of rat papilla. Higher magnification of same section as Fig. 2. Branching collecting ducts stain well. Numerous elongate cells with processes are arrayed in parallel rows in the interstitial tissue. Cells are oriented at right angles to the direction of the collecting ducts.

are briefly (0.5 to one minute) flushed with N_2 and then incubated at 37.5°C for from 0.5 to 2 hours. They are washed in 4% formaldehyde, 1% acetic acid and then H_2O and mounted in glycerine jelly.

In hundreds of slides the staining patterns of rat kidney proved to be highly reproducible and were distinctly different for each of the 2 diaphorases and for the succinic dehydrogenase system. For example, the renal papilla, unstained with the succinic dehydrogenase system, shows intense staining of the entire length of the collecting tubules with DPN diaphorase (Fig. 1,3). On the other hand only the terminal portions of the collecting tubules in the papilla stain deeply with TPN diaphorase (Fig. 2). The thick ascending portions of Henle's loop seen in the outer medullary zone, stain intensely both with the succinic dehydrogenase system and DPN diaphorase (Fig. 4), but less intensely with TPN diaphorase (Fig. 5). In contrast, the thin limbs of Henle stain brilliantly only with TPN diaphorase (Fig. 5). With this method thin limbs, so difficult to visualize with conventional histologic methods, are beautifully delineated. Capillary endothelium as well as glomerular tufts, virtually unstained with succinic dehydrogenase system, are delicately stained with TPN diaphorase, and to a lesser extent with DPN diaphorase. Similarly epithelium of the renal pelvis shows staining with both TPN and DPN diaphorase.

In sections stained for TPN diaphorase, rows of darkly stained fusiform and stellate cells are seen in the distal half of the papilla. These cells are oriented transversely to the direction of the collecting ducts, thin limbs and capillaries (Fig. 7). Work is in progress to determine whether these cells have contractile properties and whether they play any part in the regulation of urinary flow.

Summary. Methods for the specific staining for DPN diaphorase and TPN diaphorase in frozen sections of fresh tissue are presented. The staining patterns of rat kidney are highly reproducible and are distinctly different for each of the 2 diaphorases and for the succinic dehydrogenase system.

We wish to thank the Hercules Powder Co. and Dow Chemical Co. for supplying samples of the cellulose derivatives.

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Combined Use of Ultraviolet Irradiation and Beta Propiolactone Sterilization of Sera Infected with a Virus.* (21157)

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The transmission of serum hepatitis by blood and blood products presents a vexing and serious problem. Physical (ultraviolet) and chemical methods (B-propiolactone, etc.) have been utilized in efforts to sterilize plasma and serum and it would appear that these technics have some value in reducing the incidence of hepatitis resulting from infusion of these products. However, they are frequently not completely sterilized by any method used currently as was shown in recent clinical tests(1-3).

The purpose of this communication is to describe a method of treatment which seems to offer a rationale for use. Orienting experiments have shown that either ultraviolet treatment or beta propiolactone resulted in the death of *almost* all of the micro-organisms. The problem then would involve the inactivation of the relatively few micro-organisms which survive the first type of treatment. An analogy may possibly be found in the field of chemotherapy where the combination of drugs has been used to combat diseases in which minute numbers of the etiologic agent are refractory to one drug but succumb to another drug which has a mechanism of action different from the first drug. It was decided, therefore, to utilize ultraviolet irradiation to destroy the bulk of virus particles (bacteriophage) added to serum, followed by beta propiolactone in order to kill the relatively few survivors. This sequence was chosen since it was not known whether ultraviolet irradiation would have any effects on the beta propiolactone although the reversal of the sequence might be perfectly satisfactory.

Materials and methods. The suspending fluid used in all of the experiments was normal human serum. The sera were kept frozen until the day they were to be used at which time they were thawed and filtered through a Seitz sterilizing pad and the bacteriophage was added directly before treatment in order to give the concentrations shown in Tables I and II. The virus used was the T4r coliphage furnished through the generosity of Dr. S. S. Cohen.

The ultraviolet source was the standard Dill apparatus† and the method recommended by the National Institutes of Health was employed in which the rate of flow is about 250 ml per minute. The B-propiolactone was supplied through the courtesy of Dr. George H. Mangun. The treatment was carried out at room temperature.

Results. Table I shows that a minute number of virus particles escaped the effects of ultraviolet irradiation or of beta propiolactone when used separately but that when the two methods were employed in sequence complete sterilization was effected.

One of the major criticisms concerning the use of chemical agents for sterilization of plasma or serum is the possibility of deleterious action on the proteins. With this in mind it was thought advisable to determine the minimal concentration of beta propiolactone that would be efficacious. The results presented in Table II show again that small numbers of virus particles remained viable following ultraviolet irradiation but that sterilization was obtained with 1.5 g beta propiolactone per liter of serum when it is added after the ultraviolet treatment.

We are indebted to Prof. Alfred Chanutin for the electrophoretic analyses of serum before and after treatment with the combined therapy of ultraviolet irradiation and 1.5 g of beta propiolactone per liter. The patterns were identical showing no changes in the serum proteins as determined by this technic.

* Presented in part on April 7, 1954, before the Panel on Sterilization of Blood and Plasma of the National Research Council.

† Manufactured by J. J. Dill Co., Kalamazoo, Mich.

TABLE I. Effect of Ultraviolet Irradiation, Beta Propiolactone and the Combination of These Two Agents in Serum Contaminated with Bacteriophage.

	Original No. phage particles per ml serum*	Treatment	Phage counts/ml serum after treatment 15 min.	1 day	2 days	6 days
1.	560×10^6	UV†	990		300	170
2.	560×10^6	UV + BPL‡		0	0	
3.	494×10^6	BPL‡	210×10^6		49×10^4	30
4.	56×10^6	Control			30×10^6	9×10^5
5.	100 ml of #1 after UV	BPL‡			0	

* 200 ml normal human serum (Seitz filtered) per bottle.
† UV apparatus used was standard Dill machine. Aerogenes control was completely killed.
‡ BPL—B propiolactone (3 g/liter). In #2 BPL added 15 min. after UV.

The results reported here based on a combined method of treatment of virus infected serum would appear to be promising and certainly worthy of further consideration, particularly since the type of ultraviolet apparatus used here is already extensively in use throughout the United States and since the subsequent step of adding beta propiolactone is relatively simple.

TABLE II. Effect of Varying Amounts of Beta Propiolactone following Ultraviolet Irradiation in Serum Contaminated with Bacteriophage.

Sample	Phage counts/ml serum Before UV	After UV
A	876×10^6	1070
B	944×10^6	340

A and B divided into 4 equal portions as follows:

	BPL* in g/liter	16 hr phage counts/ml of serum		BPL* in g/liter	16 hr phage counts/ml of serum
A 1	3	0	B 1	1.5	0
A 2	1.5	0	B 2	.19	90
A 3	.75	20	B 3	.05	160
A 4	.38	110	B 4	0	120

* Beta propiolactone added about 30 min. after UV.

Aerogenes control 3.5×10^6 was completely killed by UV.

Conclusions. Serum heavily infected with a virus (bacteriophage T4r) was sterilized by a combined method of treatment which consisted of ultraviolet irradiation followed by the addition of beta propiolactone. The beta propiolactone in concentrations as low as 1.5 g per liter could be used effectively. Under these conditions there is no change in the serum proteins as determined by electrophoretic analyses. Under the conditions in this study, either ultraviolet irradiation or beta propiolactone individually did not sterilize the serum.

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Electrophoretic Fractionation of Serum Protein in Multiple Sclerosis.* (21158)

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Aberrations in blood proteins in multiple sclerosis have been reported previously. Swank, Franklin and Quastel(1) have observed abnormal chromatograms of plasma proteins in the acute phase of multiple sclerosis, and Putnam(2) found lowered fibrinogen and an increase in the euglobin fraction. In an investigation of a series of neurological disorders, with no information as to the specificity of the disease, Fisk, Chanutin and Klingman(3) found a decrease in the plasma albumin concentration in this general grouping. In contrast, numerous investigators of whom Dobin and Switzer(4) are the most recent, have reported no abnormal plasma protein findings. In view of the lack of any definitive investigation of serum proteins in this syndrome employing electrophoresis, it was considered advisable to obtain such information.

Methods and apparatus. Fasting blood samples were obtained from patients where a diagnosis of multiple sclerosis had been made by the neurological consulting staff of the Veterans Administration Hospital. The cases were selected at random, with the exception that the older age group was omitted, and our patient population ranged from 27-41 years. Normal subjects were obtained from the laboratory and resident staff. Twenty-seven patients and 16 controls were studied, and since no correlation was observed between the disease state and the findings both quiescent and acute cases are grouped together.

The electrophoretic separation was carried out in a Perkins-Elmer Model No. 38 Tiselius apparatus after preliminary comparisons with the Klett apparatus had substantiated the observations of Moore and White(5) that the 2 instruments gave results that were in good agreement. Determinations were carried out in a veronal buffer at pH 8.6 and 0.1 ionic strength, after 24-hour dialysis against the buffer at 3°C, at a protein concentration of approximately one percent. Fractionation was completed after 70 to 80 minutes. Initial and final boundaries were obtained by the Longsworth scanning technic(6). Area and mobility measurements were made on the ascending boundary. Since all mobility measurements were within normal limits the data have been omitted.

Protein determinations were obtained by the micro-Kjeldahl method and nitrogen values converted to protein using 6.25 as a factor.

Results. A statistical analysis of the results obtained are presented in Table I. A comparison of the normal and pathological groups reveals that the latter show a diminished albumin component and a concomitant increase in the α_2-globulin component. The albumin fraction averaged 55.0% in the multiple sclerosis cases and 60.2% in normals, while the α_2-globulin component averaged 12.4% and 8.9% in the respective groups, the P value in both cases being less than 0.001, showing a high degree of significance for the differences. The other globulin components showed no significant differences between the two groups. Because of the diminished albumin and increased α_2-globulin component in the pathological group the A/G ratio was decreased in this group, averaging 1.25 as compared to the normal value of 1.52. This difference was statistically significant ($P < .001$).

Protein determinations showed average values of 7.36 and 7.20 for the multiple sclerosis patients and normals respectively, the differences not being statistically significant.

In addition to the quantitative variations found in the pathological group, a qualitative difference in the α_2-globulin component was also observed. In 15 of the 27 patients, the α_2-globulin component exhibited a "double"

* This work has been aided by a grant from the Multiple Sclerosis Foundation of America. Dr. Lewis J. Pollock, responsible investigator.

TABLE I. Statistical Data of Results of Serum Protein Fractionation in Multiple Sclerosis.

	Total protein, g/100 ml		Electrophoretic distribution of protein fractions											
			Albumin, %		α_1-globulin, %		α_2-globulin, %		β-globulin, %		γ-globulin, %		A/G	
	Path.	Normal	Path.	Normal	Path.	Normal	Path.	Normal	Path.	Normal	Path.	Normal	Path.	Normal
Mean	7.36	7.20	55.0	60.2	4.9	4.7	12.4	8.9	14.7	14.4	13.1	11.7	1.25	1.52
Stand. deviation	.73	.43	4.71	2.35	.27	.23	2.81	1.26	2.60	1.63	3.82	2.58	.25	.15
Stand. error of mean	.19		1.08		.08		.62		.64		.98		.06	
t value	.85		4.81		.003		5.60		.46		1.43		4.43	
P	.40		<.001		—		<.001		.65		.25		<.001	

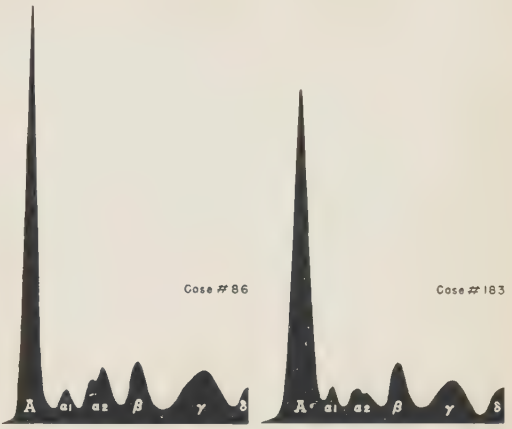

FIG. 1. Electrophoretic patterns of 2 cases of multiple sclerosis showing "double" α_2-globulin peak (ascending boundary).

α_2-globulin peak, not commonly demonstrated in normal patterns. Two examples of this "double" peak are illustrated in Fig. 1. No correlation between the existence of this heterogeneity in the α_2-globulin component and its magnitude was observed. Our lowest α_2-globulin value in the patient group exhibited this disturbance in the peak.

Discussion. Deviations from the normal electrophoretic pattern of blood proteins have been demonstrated in a variety of pathological conditions. Changes in all components in disease states have been recorded, and though an increase in γ-globulin in an inflammatory process, presumably due to antibody production, is fairly general, other changes are less well characterized. Some rationale for a hypoalbuminemia has also been evolved, but changes in the α- and β-globulin components are non-specific and less well understood. The high degree of association of lipid material in these 2 protein types might implicate some aberration in lipid metabolism with an abnormality in these components, but as pointed out earlier, such correlations have not been established. Some of our own results on cholesterol and total phospholipids(7), provide no evidence for any abnormal blood levels of these fractions in this syndrome.

Despite the extensive application of electrophoretic technics to disease states the neurological disorders have not been generally investigated, and it is of interest that an atypical

pattern is obtained in this study. Routh and Paul(8) in a similar study on anterior poliomyelitis obtained electrophoretic patterns which bear some similarity to our cases of multiple sclerosis. The similar distribution of protein fractions in these 2 dissimilar types of neurological lesions is to be noted, and the systemic change manifested by an increased α_2-globulin fraction, exhibits a uniformity which may be significant, and such studies should be extended to other types of neurological disturbances. The fact that a biochemical aberration can be demonstrated in the blood in neurological disorders is of some importance in further investigations on the behavior of nervous tissue. While this aberration is probably secondary in nature, the causative elements of such a finding should not be ignored.

Summary. 1. Electrophoretic fractionation of serum proteins in 27 cases of multiple sclerosis showed an increase in the α_2-globulin moiety with a concomitant reduction in the albumin component. This resulted in a lowered A/G ratio in the pathological cases. These differences were statistically significant. 2. In 15 out of the 27 cases an electrophoretic heterogeneity in the α_2-globulin fraction was observed which was characterized by a "double" peak.

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Association of Group A Streptococci with an Outbreak of Cervical Lymphadenitis in Mice. (21159)

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In October, 1953 a suppurative involvement of the neck was observed in Princeton mice by Dr. Alice E. Moore at the Sloan-Kettering Institute. These animals had been injected subcutaneously some weeks earlier with bacteriologically sterile suspensions in connection with her work on the suppression of tumor growth by viruses. Since the cervical reaction was in no way related to the activity of the injected agents a number of the affected mice were brought to the laboratories of animal pathology at the Rockefeller Institute for examination. The Princeton strain, which was originally established in these laboratories, has been under observation for many years. The mice used by Dr. Moore were obtained, however, from a commercial colony.

The signs of disease were unlike any previously observed in Princeton mice. The under surface of the neck showed eroded areas, with loss of hair and matting of the hair by a sticky exudate. In some animals the cervical lymph nodes were prominent and palpable. The mice were sickly in appearance and several that were held under observation died within a few days.

Fifteen of the diseased mice were killed and autopsied. The diagnosis in all of them was purulent cervical lymphadenitis. It resembled the condition originally described in guinea pigs by Boxmeyer(1) and sometimes known as "lumps" but was less progressive. In advanced cases one or more of the abscessed cervical nodes ruptured, releasing a semi-fluid purulent exudate. Its volume was evidently increased by subsequent involvement of the

adjoining tissues. Perforation of the skin ultimately occurred with drainage of the fluid through a clean-edged hole.

A number of the mice also showed unmistakable evidence of infectious catarrh, and pleuropneumonia-like organisms (PPLO) were isolated from the middle ears. In addition, these organisms were recovered from the external cervical exudate of 2 animals, presumably as secondary invaders acquired by contact.

Stained films of exudate from the involved lymph nodes invariably showed gram-positive cocci and colonies of a hemolytic streptococcus were regularly obtained on horse blood-agar plates. Dr. Rebecca Lancefield kindly identified the organism and found it to be a group A streptococcus. It did not belong, however, to any of the known types on the basis of the M precipitin reaction.

The experimental production of streptococcal cervical lymphadenitis in mice was reported by Sonkin(2). He described a technic for the deposition of inocula on the nasal mucosa in such a manner that they were confined there and did not flow to other portions of the respiratory tract. The introduction of group C streptococci by this method was attended by a high rate of cervical lymph node inflammation, 98% in 161 mice. On direct pulmonary injection, by way of the trachea, the rate was much reduced, 2.3% in 42 mice. A fatal pneumonia occurred in all of the animals of both series. Glaser, Berry, and Loeb (3) employed a modification of this method with group A streptococci. Their mice were first injected intraperitoneally with chloral hydrate and then anesthetized with ether. They also obtained a high rate of cervical lymphadenitis. The infection was generally fatal within 120 hours. Webster's selected strain of Swiss mice was used in both of these investigations.

Methods. The pathogenicity of the group A streptococcus obtained from mice was determined by the nasal introduction of recently isolated pure cultures. Princeton and Swiss mice (the original, unselected, Rockefeller Institute strain) were used in these tests. Most of the injections were made in weanlings, 4 to 5 weeks old (10-15 g in weight), but adults, 8 to 12 months old (25-50 g in weight) were also employed.

The streptococcus was generally isolated from lymph node exudate and transferred in culture not more than 5 times. It was grown in 1.0 ml of 10% horse serum-heart infusion bouillon (pH 7.4) at the base of slanted nutrient agar. Six- to 7-hour growths at 37°C were generally used but occasionally the culture was incubated up to 18 hours. The fluid was removed and diluted with an equal volume of saline. The slant was reincubated and then preserved as a stock culture.

The mice were anesthetized with ether in an open battery jar. Prior injection of chloral hydrate was also tried but was not superior to ether alone. The inoculum was introduced by one of 2 methods referred to as nasal inhalation and nasal deposition. The first method entailed placing about .02 ml of diluted culture dropwise on the nares of weanlings and up to .05 ml with adults. A Luer syringe (¼ ml) with a 24-gauge needle was used. With the second method a special 30-gauge needle delivering a minute drop, .01 ml or less, was employed. The needle was inserted a short distance into one nasal passage and a single drop expelled. This method was less precise than that used by Sonkin(2) but serviceable. The 6- to 7-hour cultures of the streptococcus contained between 6×10^8 and 8×10^8 organisms per ml.

The mice were generally injected in groups of 5, either sex being used. Most of the animals that died were autopsied. All survivors were killed after the 7th to the 14th day and examined. When indicated, Gram-stained films and blood agar cultures were made from the lungs and lymph nodes. The middle ears and nasal passages of all survivors were exposed and aspirated with a capillary pipette. If exudate was present, blood agar cultures and films were made. In a few instances 20% horse serum-agar plates containing 2500 units of penicillin were also inoculated for PPLO determination.

Results. The experimental findings, summarized in Table I, indicated a difference in the susceptibility of Princeton and Swiss weanlings to the streptococcus. These 2 mouse strains are known to vary in their re-

TABLE I. Response of Princeton and Swiss Mice to Nasal Introduction of Group A Streptococcus.

Strain and age of mouse	Method of introduction	No. of mice	No. of deaths*	No. with adenitis
Swiss weanlings	Inhal.†	25	9	2
Princeton "	"	"	23	0
Swiss "	Depos.	"	1	2
Princeton "	"	"	2	18
Swiss adults	Inhal.	"	20	2
Princeton "	"	"	21	2

* All deaths occurred between the 2nd and the 7th day.

† Inhal. = Inhalation. Depos. = Deposition.

sponse to other microbial agents, as the virus of murine hepatitis(4). Princeton weanlings infected by nasal deposition were the only mice in which cervical lymphadenitis was reproduced with any degree of regularity. The morbidity rate was 72%, in comparison with 8.0% in Swiss mice infected in the same way. The reaction was commonly unilateral, corresponding to the site of deposition. It varied from focal inflammation to nearly complete replacement of the node by a purulent exudate. Release of the exudate by rupture left a thin shell of lymphoid tissue and was ultimately followed by perforation of the skin. Hemolytic streptococci were commonly recovered in pure culture from the involved nodes. The morbidity rate was considerably less than that reported by Sonkin(2) for group C streptococci but supported his conclusions on the efficacy of nasal deposition in localizing the organisms.

Nasal inhalation was more commonly followed by carriage of the streptococci to the lung. The resulting pneumonia was diffuse in distribution, often involving parts of all 5 lobes. Stained films of lung tissue showed innumerable gram-positive cocci and hemolytic colonies were obtained on blood agar. The mortality rate was much higher in Princeton than in Swiss weanlings, 92 as compared with 36%. Immature and adult Princeton mice were about equally susceptible to pneumonia. In Swiss mice, however, the adults showed a higher death rate than did the weanlings. The adult mice available were too few in number to determine the effect of age on the outcome of nasal deposition.

In connection with long continued studies, in these laboratories, on murine otitis media caused by PPLO it was of particular interest that group A streptococci also tended to involve the middle ears. In the present series otitis media was rarely accompanied by rhinitis, as it commonly is in mice infected nasally with PPLO. Only 3 mice, all adults, showed unmistakable inflammation of the nasal passages. Middle ear examination was restricted to mice that survived at least 7 days after nasal inhalation or deposition. The incidence of otitis media in 45 Princeton weanlings was 62% (28 cases) and in 55 Swiss weanlings 72% (40 cases). Streptococci were demonstrable in exudate films or cultures from all but 2 of these mice. Two Princeton mice of this series, which includes animals not listed in Table I, and 6 Swiss were "twisters." Under usual circumstances this condition is rarely seen in mice. The head is inclined to one side; the affected animal tends to circle; and spins violently if picked up by the tail.

Five contact experiments were carried out by placing normal mice in the same cage with infected ones. If the infected animals survived, contact was maintained for at least 10 days. Two out of 15 Swiss weanlings, thus exposed, showed otitis media at autopsy. Hemolytic streptococci were recovered in pure culture from the middle ear exudate. The cervical lymph nodes and lungs of these mice were uniformly normal. Five Princeton weanlings, similarly exposed, were normal throughout. In these experiments infection was established by the nasal injection of streptococcus cultures. Two of 3 Princeton weanlings exposed to 3 naturally infected mice developed typical cervical lymphadenitis with positive cultures. In one animal an abscessed lymph node ruptured and the released exudate drained outwards through the perforated skin. One of the originally infected mice, in this series, died and was partially eaten.

Discussion. Group A hemolytic streptococci are tacitly regarded as peculiar to man and the isolation of the present type from naturally infected Princeton mice is an unusual occurrence. The source of this infection is undetermined. Hemolytic streptococci of the usual type have rarely been encountered in mice from the parent colony. The group A

type was not demonstrable in the nasal passages of adults and weanlings obtained directly from the commercial colony. Streptococci were certainly not present in the inocula which the mice received prior to the outbreak of lymphadenitis. Though direct evidence is lacking it may well be that the organisms were originally transmitted to the mice, by way of the nasal passages, from an infected attendant. What effect the preceding experimental treatment may have had on the mice is also uncertain. It is possible that they were more receptive to streptococcal infection than normal mice of the same age. The manifestations of the naturally acquired disease were fully reproduced, however, in the absence of the agents originally employed. Initial establishment of the streptococci in mice of different age groups was probably attended by a limited transmission by direct contact within each cage.

The experimental findings agreed essentially with the earlier results of Sonkin(2), and of Glaser, Berry, and Loeb(3) on the production of murine cervical lymphadenitis by hemolytic streptococci. There was a marked disparity, however, in the death rates resulting from the nasal deposition of the organisms. The susceptibility of the mice and the pathogenicity of the streptococci are variables that must be here considered. The present results were indicative of differences in the response of Princeton and unselected Swiss mice to streptococcal infection. Both of these strains differ genetically from Webster's selected Swiss strain that was used by the earlier workers. The several types of streptococci used in the current and the preceding series vary in their antigenic composition and probably differ in virulence.

Summary. Group A hemolytic streptococci were regularly isolated from the abscessed lymph nodes of Princeton mice during an outbreak of cervical lymphadenitis. The naturally acquired disease was reproduced in normal Princeton weanlings by nasal deposition of the organism in pure culture. The mortality rate was markedly increased, however, by nasal inhalation of the inoculum. In Swiss weanlings the morbidity rate of cervical lymphadenitis after deposition and the mortality rate after inhalation were much reduced. Nasal introduction of the streptococcus was attended by otitis media in 28 out of 45 Princeton wealings (62%) and 40 out of 55 Swiss (72%).

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Protective Action of Tetanus Toxoid Unrelated to Active Immunization in Mice.* (21160)

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Recent studies by us, as well as by others, indicate clearly that an individual previously immunized with tetanus toxoid enjoys a long-continued protection against the action of tetanus toxin, not only in respect of serum antitoxin levels, but in the ability to respond rapidly to a booster dose of toxoid. Even 10 to 11 years after the last immunizing injection, most persons respond to a booster dose of toxoid with a detectable rise in serum antitoxin within 3 to 5 days(1). While the brief lag period in antitoxin response after a booster dose of toxoid probably does not indicate lack of protection, nevertheless it might be useful to find means of supplementing the individual's immunity during this period other than

* Supported by a grant from the Whitehall Foundation.

by the injection of antitoxin, which when contained in horse serum leads to undesirable reactions in a high proportion of patients.

Krech(2), and Lemetayer, Raynaud, and their associates(3-5) found that tetanous toxoid administered to mice inhibited the action of tetanus toxin given 24 hours later. The present study is an extension of those observations.

Procedure. Highly purified tetanus toxin (Lot No. LCI 10-9-47) prepared by Dr. Louis Pillemer of Western Reserve University was used in a dose which varied between 1 mouse LD_{50} and 1 mouse mld. This dose regularly caused severe symptoms or death. The toxin used in each experiment was removed from –40°C, thawed, and diluted to proper strength in broth of pH 6.6 just prior to injection. Therefore, each experiment was performed with toxin of equivalent potency except for slight unavoidable dilution inaccuracies. That the variation in toxin strength was slight can be noted by comparison of groups of mice that received the same dose of toxin in different experiments. The toxin was contained in a volume of 0.2 ml and was injected into mice weighing 18 ± 2 g intramuscularly at the base of the tail on the right.

Symptoms were graded as follows:

Slight. Bulging of the left side of the abdomen because of spasm of the abdominal muscles on the right; sluggishness of right hind leg.

Severe. Spastic deflection of the tail to the right and paralysis of the right hind leg, often progressing to an inability of the mouse to right itself when placed on its back, and convulsions.

In severely paralyzed mice deaths occurring after the first week may have been due to lack of nourishment. Mice that showed severe symptoms but did not die recovered completely after many weeks.

Commercially available fluid tetanus toxoid was used in these experiments.† The toxoid, except when combined *in vitro* with toxin, was injected intramuscularly at the base of the tail on the left.

† The toxoid was supplied by the Lederle Laboratories Division, American Cyanamid Co., under the designation Tetanus Toxoid Fluid-Purogenated.

TABLE I. Effect of Varying Dose of Toxoid when Toxin and Toxoid Are Administered Simultaneously.

Dose of toxoid, ml	Deaths		Severe symptoms	
	Days after inj. of toxin			
	5	9	5	9
None	20/20*	—	20/20†	—
.2	1/20	15/20	20/20	20/20
.4	0/20	2/20	5/20	4/20
.8	0/20	0/20	1/20	1/20

* Numerator: No. of deaths. Denominator: Total No. mice inj.

† Numerator: No. with severe symptoms including death. Denominator: Total No. mice inj.

Results. Effect of varying dose of toxoid—toxin and toxoid administered simultaneously. When the toxoid was administered simultaneously with, or a few moments prior to the injection of toxin substantial protection was noted with each of the doses of toxoid employed, namely 0.2, 0.4, and 0.8 ml, as compared with mice which received no toxoid. This protective effect, which bore a direct relation to the size of the dose of toxoid, was somewhat greater in terms of survival than in terms of the absence of symptoms (Table I).

Effect of varying the interval between toxoid and toxin injection. Experiments were undertaken to investigate the effect of giving 0.4 ml of toxoid at varying intervals before the toxin. A high degree of protection is afforded mice given toxin and toxoid simultaneously, and somewhat less protection is afforded mice given toxoid 24 hours prior to the injection of toxin (Table II). Only slight protection was noted when the interval between toxoid

TABLE II. Effect of Varying Interval between Toxoid and Toxin Injection. Dose of toxoid 0.4 ml.

No. of days prior to inj. of toxin that toxoid was given	Deaths		Severe symptoms	
	Days after inj. of toxin			
	5	10	5	10
No toxoid	8/10*	10/10	10/10†	10/10
7	0/10	3/10	6/10	6/10
5	7/10	9/10	10/10	10/10
3	5/10	10/10	10/10	10/10
1	0/10	8/10	8/10	8/10
0	0/10	0/10	5/10	2/10

*† See footnotes, Table I.

TABLE III. Effect of Divided Doses of Toxoid at Daily Intervals.

	Deaths		Severe symptoms	
	Days after inj. of toxin			
	5	10	5	10
Group A	0/10*	5/10	3/10†	6/10
B	1/10	4/10	3/10	9/10
C	0/10	4/10	4/10	8/10
D	0/10	3/10	3/10	9/10
E	7/10	10/10	10/10	10/10

*† See footnotes, Table I.

and toxin injection was 3 days, and no protection was noted when the interval was 5 days. Protection exhibited by the mice receiving toxoid 7 days prior to the injection of toxin is taken as a manifestation of early active immunization.

Effect of divided doses of toxoid. The same dose of toxoid, namely 0.4 ml, was given in divided doses of 0.1 ml per day according to the following protocol. The day the toxin was given is referred to as zero day, and the minus and plus signs indicate days before or after zero day. All groups received the standard dose of toxin as described under Procedure.

Group A: 0.1 ml toxoid on −3, −2, −1, 0; Group B: 0.1 ml toxoid on −1, 0, +1, +2; Group C: 0.1 ml toxoid on 0, +1, +2, +3; Group D: 0.4 ml toxoid on day 0 only; Group E: No toxoid.

Each group that received divided doses of toxoid had significant protection similar in degree to the protection afforded mice receiving the same total dose (0.4 ml) on zero day (Table III). Moreover, there is no significant difference in the protection afforded groups A, B, and C. The results of this experiment should be viewed in relation to the previous one, since a single large dose given 3 days before the toxin seems to have less protective effect than divided doses given daily over the same period.

Effect of mixing toxoid and toxin in vitro prior to injection. When the standard dose of toxin was mixed with 0.4 ml of toxoid *in vitro* and the mixture immediately injected into mice intramuscularly at the base of the tail on the right the protection appeared to be greater than that afforded by the injection of toxoid at a site opposite that used for the injection of toxin. This effect was more apparent in terms of absence of symptoms than in terms of survival. Tetanus toxin mixed with diphtheria toxoid was used as a control and no protection was found (Table IV). Another experiment in which the toxin dose was very small yielded similar results.

Discussion. In addition to the well-known immunizing action of tetanus toxoid, these experiments and those of the authors previously cited, suggest that toxoid also inhibits the action of toxin by a mechanism not related to antibody formation. The experiments throw no direct light on the nature of this inhibitory action. Harvey(6) has shown that tetanus toxin is capable of peripheral action in the region of the neuromuscular junction, and postulates that the toxin affects the motor nerve ending with resulting derangement in the acetylcholine-cholinesterase relationship. It may be that these action sites of tetanus toxin have an equal affinity for toxoid, which when present occupies a proportion of the normal receptor sites thus serving to block the action of toxin. On the other hand, it is possible that the combination of toxoid and toxin results in inactivation of the latter.

Regardless of the underlying mechanism involved, it appears that in our experiments the toxoid was transported via the blood stream to the site into which toxin had been injected. It might be expected that the closer the toxoid can be approximated to the toxin depot, the more effective would be the protective value

TABLE IV. Effect of Mixing Toxoid and Toxin *In Vitro* Prior to Injection.

	Deaths		Severe symptoms	
	Days after inj. of toxin			
	5	10	5	10
No toxoid	5/6*	5/6	5/6†	5/6
Tetanus toxin + tetanus toxoid	0/6	1/6	0/6	1/6
Tetanus toxin + diphtheria toxoid	2/6	6/6	6/6	6/6
Tetanus toxin and tetanus toxoid at opposite sites	0/6	2/6	5/6	6/6

*† See footnotes, Table I.

of the toxoid, since toxoid could reach the sites of critical action by direct diffusions as well as through the blood stream. The results of the experiment in which toxoid and toxin were combined *in vitro* suggest that this consideration may be playing a role. Similarly, Raynaud, *et al.*,(4) reported that when toxoid and toxin were injected into the same leg local tetanus did not develop, although local tetanus did develop if the injections were made into opposite legs.

Another observation worthy of emphasis is the efficacy of small daily doses of toxoid in inhibiting the action of toxin. Injection of 0.1 ml of toxoid daily for 4 days commencing 3 days before, 1 day before, or on the day of toxin administration resulted in protection similar in degree to that afforded by the injection of the same total amount of toxoid, namely 0.4 ml, simultaneously with the injection of toxin, and the protection was substantially greater than when a single dose of 0.4 ml was given 3 days before the toxin injection. The fact that protection from small divided doses was just as great when the toxoid injections were started coincident with the injection of toxin, as when toxoid was begun 3 days before, would seem to eliminate the possibility that an accelerated active immune response was playing a role.

It should also be emphasized that the doses of tetanus toxoid used in these experiments were very large in relation to the body weight of the mouse, and it is not known to what extent these observations can be interpreted in terms of the problem in human beings. Certainly, this whole phenomenon should be explored first in larger animals.

Summary. 1. Mice injected with a single dose (0.4 ml) of fluid tetanus toxoid 24 hours prior to, or simultaneously with a small dose (1 LD_{50} to 1 MLD) of tetanus toxin exhibited a degree of protection against the action of the toxin. The protective effect varied directly with the dose of toxoid. Toxoid given 3 or 5 days before the toxin afforded little or no protection. 2. Small daily doses of toxoid (0.1 ml) given over a 4-day period commencing 3 days prior, one day prior, or on the day of toxin administration were as efficacious in inhibiting the action of toxin as an equivalent single large dose of toxoid (0.4 ml) administered coincident with the injection of toxin. 3. When toxoid and toxin were mixed *in vitro* prior to injection into mice, the protective effect of the toxoid appeared to be enhanced.

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Transfer to X-Irradiated Rabbits of Lymph Node Cells Incubated *in vitro* with *Shigella paradysenteriae.** (21161)

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In an earlier paper it was reported that if 4 days after the injection of dysentery bacilli into the foot pad of the rabbit, cells were teased from the lymph node draining the site of injection and transferred into fresh recipient rabbits, the latter would show detectable serum titers of agglutinins to the dysentery organisms(1). A later study explored the time interval between the injection of antigen into the donor and the collection of its lymph node cells(2). It was found that if the lymph node cells were obtained as early as 10 minutes after the injection of the antigen and transferred to X-irradiated recipients, agglutinins would appear in the recipients' sera a few days later. Because of the shortness of this interval the possibility was explored that contact between the antigen and the cells of the donor could be reduced even further, *i.e.*, to contact *in vitro*. Preliminary results of this study are reported below.

Materials and methods. Lymph node cells were obtained from the popliteal and axillary lymph nodes of rabbits weighing 2-2½ kg. These rabbits had received either no injection of antigen or a subcutaneous injection of a heterologous antigen into the fore and hind foot pads. This heterologous material, 0.2 ml of packed sheep red blood cells was injected 4 days prior to sacrifice in order to stimulate hyperplasia of the local lymph node and thereby increase the yield of cells. The lymph nodes were excised and bathed in Tyrode's solution (made up without bicarbonate) containing 0.12% gelatin. After trimming the nodes the cells were teased free by means of 2 dissecting needles. At this time the number of cells was determined by the standard method for white blood cell counts. Each group of 225 x 10^6 cells present in the suspension was selected as the amount to be injected into one recipient rabbit. Subsequently, a given amount of antigen was added according to the number of recipients involved. The state of viability of the cells was determined by a cell count for which the cells were diluted in normal rabbit serum containing 0.7% trypan blue dye. Those cells failing to take up the blue dye were considered viable, while those stained blue were considered non-viable.

For serologic testing serial 2-fold dilutions of rabbit serum were made in volumes of 0.4 ml. To these were added 0.2 ml of a 0.05% suspension of alcohol-treated *Shigella paradysenteriae*. After shaking and after one hour of incubation at 37°C, the tubes were stored at 4°C for 48 hours, and then examined for evidence of agglutination.

The recipient rabbits were irradiated 24 hours prior to cell-transfer at a dosage of 425 r whole body X-irradiation (200 Kv.; 20 ma; 67.5 cm distance from the target to the bottom of the container, yielding 18 r (air) per minute; 0.5 mm copper plus 1 mm aluminum filtration; half-value layer 1 mm copper).

Results. The popliteal lymph nodes of rabbits which had not previously been injected with dysentery bacilli were excised and trimmed, and the cells were teased free. To the suspension of cells was added a given amount of a suspension of killed dysentery bacilli (0.07 ml of a 1% suspension of organisms per 225 x 10^6 cells). The mixture was incubated at 37°C for one hour, filtered through a number 80 stainless steel mesh and washed in a large volume of Tyrode-gelatin solution. After centrifugation at 1400 RPM for 5 minutes, the supernate was removed and the sediment resuspended in 20 volumes of

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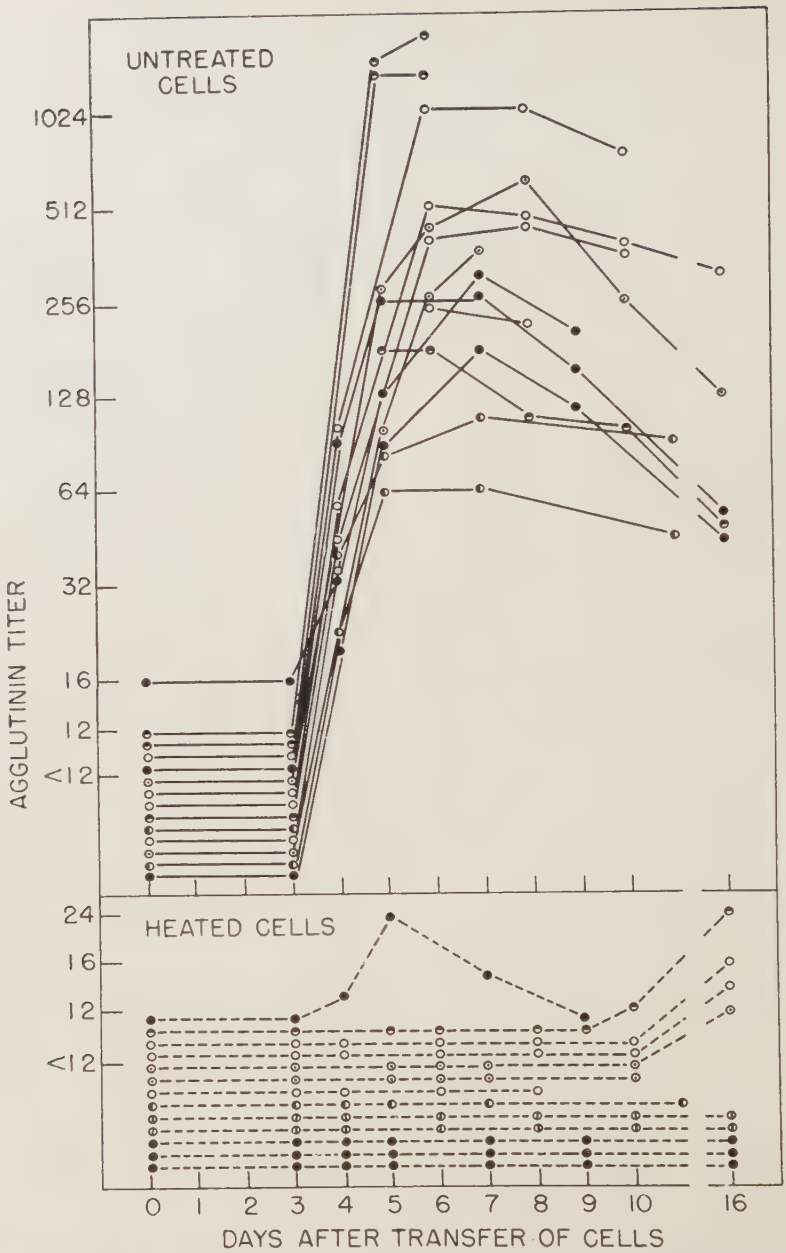

FIG. 1. Serum agglutinin titers of X-irradiated recipients of untreated and heated lymph node cells. Prior to transfer cells had been incubated *in vitro* with dysentery bacilli. Various symbols indicate different experiments. Termination of a curve before the end of the graph is due to death of the animal.

Tyrode-rabbit serum. This suspension was centrifuged at 600 RPM for 5 minutes, and the sediment resuspended in 5 additional volumes of Tyrode-rabbit serum mix. Of this cell suspension 0.9 ml was injected intravenously into each of the recipient rabbits which had received 425 r of X-irradiation 24 hours earlier. A portion of the cell suspension was heated for 60 minutes at 60°C, and of this portion 0.9 ml was injected into each of a group of recipient rabbits previously X-irradiated. Blood samples were obtained from all

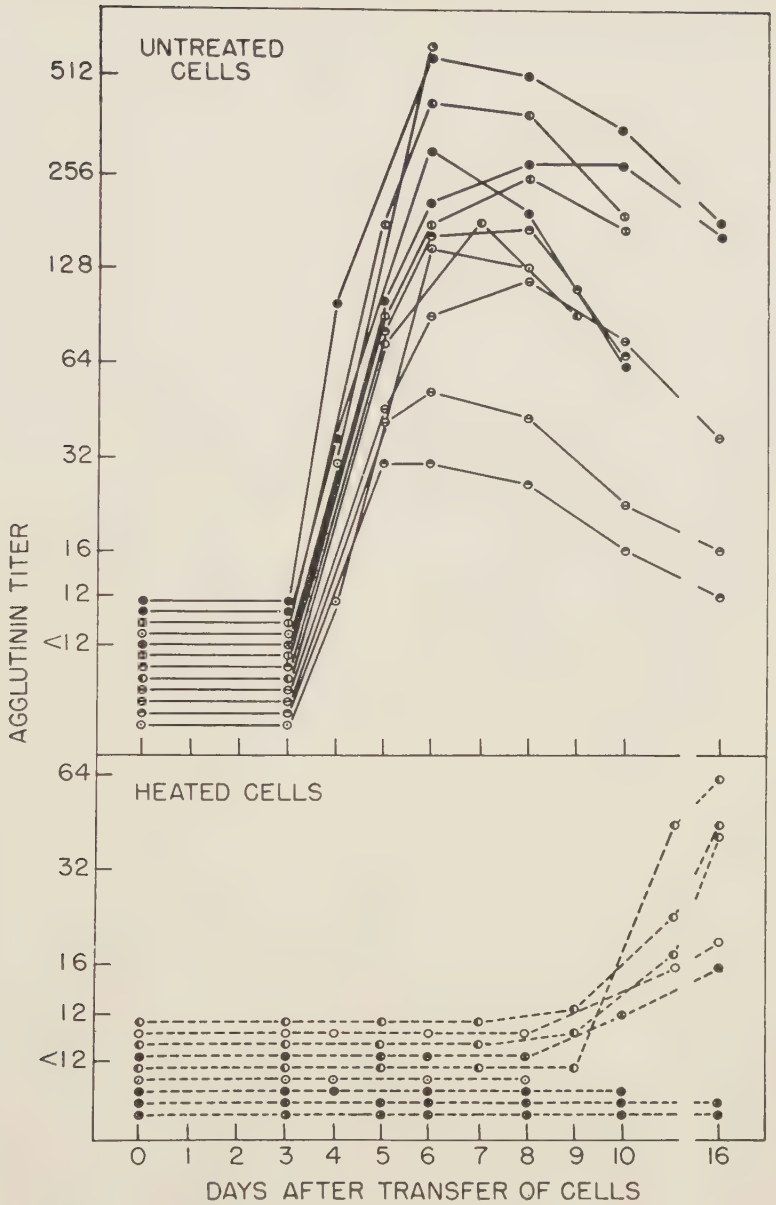

FIG. 2. Serum agglutinin titers of X-irradiated recipients of untreated and heated lymph node cells. Intravenous injection of washed cells into recipients was followed immediately by intravenous injection of dysentery bacilli into those animals.

recipients at regular intervals following the transfer of cells.

The sera of the recipients were tested for agglutinins to dysentery bacilli. No such agglutinins were detectable in the sera of these recipients up to and including the third day after transfer. On the fourth day agglutinins appeared in sera of the recipients of untreated cells. The level of this antibody increased on the fifth day and usually reached its peak by the sixth to eighth day. In the sera of the recipients of heated cells no detectable antibody appeared during the first week. Results of a group of such experiments are shown in Fig. 1.

In another series of experiments, cells de-

rived from the popliteal lymph nodes of rabbits not previously injected with dysentery bacilli were teased and suspended as for a typical cell transfer experiment (approximately 250 x 10^6 cells per ml). Of this suspension 0.9 ml was injected into the marginal ear vein of previously irradiated recipient rabbits, and this was followed immediately by injection of 1 ml of a 0.005% suspension of dysentery bacilli in the opposite marginal ear vein. The same procedure was also followed with cells which had been previously heated for 60 minutes at 60°C.

Again in this series of experiments, agglutinins appeared in the sera of the irradiated animals receiving untreated cells but not in the recipients of heated cells. Antibody generally appeared on the fourth day and thereafter followed a pattern similar to that observed in the experiments described above in which lymph node cells were incubated with dysentery bacilli. Fig. 2 shows the data obtained in typical experiments of this series.

Discussion. In the experiments described above there was no contact between the antigen and the cells obtained from the donor animal except for contact *in vitro,* and subsequently within the body of the recipient animal, or only the latter. The recipient animals had been irradiated in a dosage adequate to prevent active immunization by the amount of antigen present in the cell-suspensions injected. This was indicated by the fact that the injection of comparable suspensions which had been heated was not followed by the appearance of antibody titers of the same range in the sera of recipient animals.

It would appear, then, that antibody was produced in the recipient animals following the injection of uninjured lymph node cells which had had contact with the antigen only after removal of the cells from the tissues of the donor animal. There are at least 2 mechanisms which might account for these observations. One of these would involve a physiologic function of the transferred cells. In terms of such a hypothesis the cells would take up some form of the antigen during their incubation together *in vitro,* or subsequently during their coexistence in the blood or tissue fluids of the recipient animal, and then synthesize the antibody which subsequently appears in the serum. That sufficient contact between transferred cells and the antigenic material can occur in the body of the recipient animal is indicated by the fact that separate injection of cell and antigen into that animal can result in the production of antibody.

Another possible explanation of these data is that the transferred cells might release into the tissues of the irradiated animal some constituent or product of uninjured lymph node cells which enables the irradiated tissues of the recipient to carry out their normal function of antibody synthesis. A hypothesis of this nature has been offered by Jacobson and Robson in explanation of their data in a recent study. These authors have shown that if during 800 r or 500 r total-body X-irradiation the spleens of rabbits are shielded, left intact for 24 hours, and then removed surgically, the animals can form antibodies to antigens injected subsequently(3). The authors attribute this to a restoration of the functional capacity of the animal's antibody-forming cells by a humoral substance entering the general circulation from the originally shielded spleen during the 24-hour period prior to splenectomy. It is not possible, however, on the basis of the data presented in that study to exclude any role of cells entering the tissues of the irradiated animal from its shielded spleen.

Summary. Cells were teased from the popliteal lymph nodes of rabbits which had not been injected with dysentery bacilli. These cells were incubated with dysentery bacilli *in vitro* and transferred to X-irradiated recipients. Agglutinins were detected in the sera of such recipients on the fourth day after transfer. When the cells were heated prior to transfer agglutinins did not appear during the first week after transfer. Similar results were obtained when suspensions of lymph node cells and suspensions of dysentery organisms were injected separately into irradiated rabbits.

We are grateful to the Radiology Department of the Graduate Hospital of the University of Pennsylvania for their generosity in permitting us the use of their X-ray therapy machine throughout this study.

Irradiation was administered by Dr. J. J. Smith of that department.

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Effect of Rate of Injection of Alloxan on Development of Diabetes in Rabbits.* (21162)

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Alloxan has been a useful laboratory tool in the production of hyperglycemia and the study of various aspects of diabetes, hypoinsulinism and carbohydrate metabolism. Knowledge of its use has been summarized and reviewed(1). Early in the course of our observations of animals made diabetic by alloxan, we noted that the toxic dose was very close to the adequate dose for the production of diabetes and that there was considerable variation in the mortality rate and in the number of animals which developed hyperglycemia. On carefully evaluating our technic and after reviewing the available literature, it appeared that differences in the rate of injection might explain some of the variations which we obtained. Although some authors have made note of the rapidity of injection this factor had not been studied extensively. As a result the study to be reported was undertaken.

The relatively rapid destruction of alloxan in blood and the fact that clamping the pancreatic vessels for a period of 5 minutes after injection prevented the appearance of diabetes (2) suggested that the persistence of adequate blood levels is essential in producing the desired Beta cell destruction. The observation that abnormalities in carbohydrate metabolism may occur despite an absence of demonstrable histological change(3) suggested that variable numbers of cells in any islet might be affected at any one time, or that functional changes might occur in normal appearing cells.

Materials and methods. The rabbits employed in this study were housed in our animal quarters for 3 to 8 weeks before they were used. They were fed purina rabbit chow and allowed water *ad lib*. No preliminary starvation was employed. Alloxan monohydrate was injected intravenously in a 3.3 or 5% solution in distilled water made slightly acid so that the pH was approximately 4.5. Rate of injection was controlled by means of a constant-rate infusion pump. Alloxan was injected in doses of 75, 100 and 150 mg/kg into the marginal ear vein. The rates of injection fell into 3 groups. (1) less than one minute, generally 15-30 seconds, (2) 5-20 minutes, generally about 10 minutes, and (3) between 30 and 50 minutes. After 3-4 hours, the animals were allowed food and water and 10 cc of a 10 or 20% solution of glucose was injected subcutaneously at 2 hour intervals for approximately 12 hours, to prevent the early hypoglycemia. After 24-48 hours, observations including blood sugar, urine volume and glucose were made. Those animals dying before the above studies were made are classified as dead. Few animals died between 48 hours and one week after injections, most of these being diabetic. Those animals showing definite hyperglycemia and glycosuria were classified as diabetic. A group of animals which showed transient hyperglycemia or glycosuria, or had abnormal glucose tolerance tests were classified as questionably diabetic.

Results. The results are presented in Fig.

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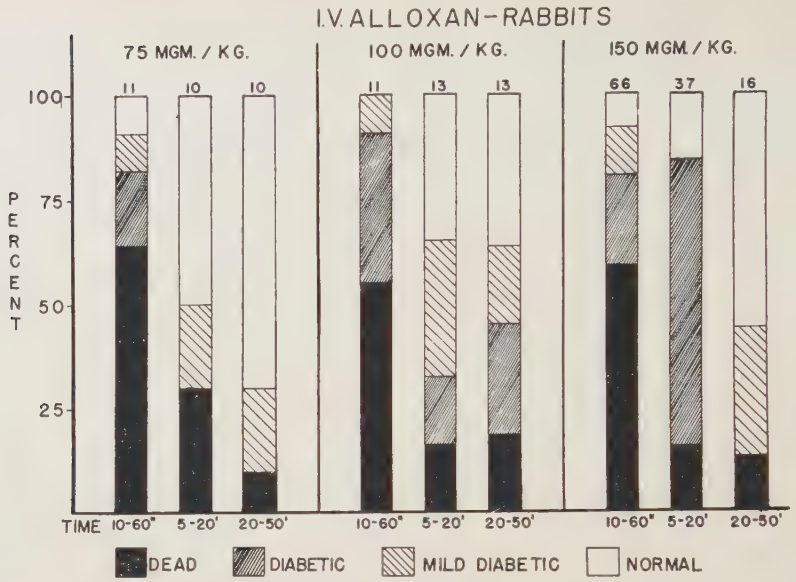

FIG. 1. Effect of rate of injection of alloxan and dose of alloxan on mortality rate and occurrence of diabetes in rabbits.

I. For each dose of alloxan the mortality rate and the number of animals with "diabetes" decreased as the rate of injection was slowed. When the alloxan in any of the doses employed was injected very rapidly, more than 50% of the animals died. When the rate was very slow, about 50% of the animals failed to show any evidence of diabetes. It would appear that a rate of injection between these extremes is most satisfactory, and when the alloxan was administered in a period of about 10 minutes, the largest number of surviving severely diabetic animals resulted.

Discussion. It would appear, from a survey of our data, that an adequate blood level of alloxan must be maintained for a certain period of time to produce regularly the desired effect on the pancreatic islets. Although we did not determine the blood levels of alloxan, it may be that the rate of destruction or inactivation of this material might differ in different individuals. Another possibility is that the blood supply to the individual islets may be intermittent, hence an adequate level of alloxan for a short time might destroy an insufficient number of Beta cells to produce diabetes.

This report was withheld for a period of almost 2 years because of the occurrence in our laboratory of a tremendous increase in mortality as a result of this treatment. We were unable to account for this by a change in diet, technic, or in the strain of animal used. For the past year, however, our results with moderately slow injection of 150 mg of alloxan per kg have again been similar to that shown in the chart. It seems obvious to us that factors other than those considered in this report must play an extremely important part in the susceptibility of the animal to this very toxic chemical. Our continued observations leave us with the distinct impression that the rate of injection as well as the dose of the drug is an important determinant in the occurrence of diabetes and survival of these animals following alloxan administration.

Conclusions. Our results indicate that the rate of injection of alloxan into rabbits is an important factor in the production of hyperglycemia and survival of these animals.

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Response to Fasting of Hepatic Arginase, Alkaline Phosphatase, and Rhodanese in Protein-Depleted Rats.* (21163)

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Lightbody and Kleinman(1) reported that there was a positive correlation between the total amount of liver arginase in the rat and the protein content of the diet. Ashworth(2) first noticed the drop in liver arginase activity on protein starvation which he interpreted as enzyme deterioration by non-use. The relation of the activity level of hepatic arginase to the protein-intake of the animal has been repeatedly confirmed(3-7). Opinions are divided, however, regarding the significance of this relationship. Miller(6) considers the changes in the arginase content of rat liver as non-specific participation in the gain and loss of hepatic protein; whereas according to Mandelstam and Yudkin(7) the relationship between enzyme production and amount of dietary protein is in conformity with the mass action theory of enzyme adaptation.

Previous studies from this laboratory(8) supported the inference that the rate of restoration of liver arginase following 70% partial hepatectomy was geared to the protein catabolism of the animals inasmuch as the rate was the faster, the higher and the more rapidly the urinary nitrogen excretion rose above the preoperative level. Accordingly restoration of arginase was fastest in protein-depleted rats in which the increase in protein catabolism is relatively greater than in protein-fed animals. The rate was enhanced by fasting and reduced by supplying non-protein calories immediately after the operation. Since the protein catabolism of the protein-starved animal can be increased by fasting to approximately the same extent as by a partial hepatectomy, it was of interest to study the response of liver arginase to fasting.

Experimental. The experiments were carried out in conjunction with assays of alkaline phosphatase previously reported by us (9). The animals were male rats from the colony of the Wistar Institute which weighed approximately 250 g at the time of transfer to the protein-free semi-synthetic diet. After 2 weeks on this diet food was withheld and groups of animals were sacrificed after 2 or 4 days of fasting for enzyme assay and chemical analysis. The methods employed have been detailed in previous publications from this laboratory(5,9).

Results. The experimental results summarized in Table I are expressed in terms of enzyme units or of mg of protein per total liver per 100 g initial body weight; *i.e.* the weight on the day of transfer to the protein-free diet. The figures represent the means and standard errors† for the control groups and the differences between the means of the experimental and control groups with the standard errors of the differences.‡ To facilitate comparison of the magnitude of the changes due to fasting the differences and their standard errors are expressed as percentages of the respective control means.

It is seen that the arginase content of the protein-depleted livers increased significantly during the period of fasting concomitantly with, though somewhat slower than the rate of urinary nitrogen excretion. The individual arginase values obtained at the 2-day interval were still within twice the standard deviation of the control group, whereas all values at the 4-day interval were beyond this range and

* This work was supported (in part) under contract DA-49-007-MD-143 between the Department of the Army and the University of Pennsylvania.

† $\pm\sqrt{\frac{\Sigma(\triangle x)^2}{n(n-1)}}$

‡ $\pm\sqrt{\frac{\Sigma(\triangle x_1)^2+\Sigma(\triangle x_2)^2}{n_1+n_2-2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}$

TABLE I. Effect of Fasting on Enzyme and Protein Content of Protein-Depleted Rat Livers.

Period	Arginase, units*	Alkaline phosphatase, units	Rhodanese, units	Protein, mg	Urinary nitrogen excr., mg/day
Control	1083 (76)† ±22.1‡	6.16 (81) ±.155	246.5 (4) ±9.1	385.7 (80) ±2.64	56.3 (4) ±2.20
2 days fast	±19.6% (4) ±9.01 P<.05	−7.3% (4) ±11.54 P>.05	+25.8%(4) ±9.75 P<.05	+10.3% (4) ±3.18 P<.01	+53.8%§(4) ±5.10 P<.01
4 " "	+57.5% (8) ±6.59 P<.01	−58.2% (8) ±8.01 P<.01	+27.5%(4) ±6.82 P<.01	+2.8% (8) ±2.62 P>.05	+73.2% (4) ±13.21 P<.01

* Units = μM of reaction product formed per min. at 25°C and pH 9.5 for arginase, at 37.5°C and pH 9.4 for phosphatase, and at 20°C and pH 7.47 for rhodanese.

† No. of experiments in parentheses.

‡ Stand. error of the mean.

§ Mean of the increments obtained in individual animals ± stand. error. Increments computed from the avg excretion rates for days 1 and 2 and days 3 and 4 respectively.

their mean significantly higher than that at the earlier interval.

The results of simultaneous assays of hepatic alkaline phosphatase show that the fall in the phosphatase content was the mirror image of the rise in arginase content. Since previous studies from this laboratory indicated that the activity levels of these 2 hepatic enzymes were affected by the metabolic state of the animal, some of the livers were assayed for rhodanese which did not display such a dependence. It is evident that the rhodanese content§ of the depleted livers increased on fasting although the increment was smaller and the maximum reached earlier than with arginase. It is of interest to notice that the protein content of the depleted livers did not diminish on fasting and even appeared to be slightly augmented at the second day interval. The tendency of the liver protein to increase during fasting in the protein-starved animal is in analogy to the high initial rate of hepatic protein synthesis following partial hepatectomy in this type of animal (8). It presumably results from the circumstance that in the state of protein starvation any extrahepatic protein breakdown raises the amino acid concentration in the liver above the level with which the "labile" liver cytoplasm was in equilibrium.

To find out whether the qualitative and quantitative differences in the response of the 3 enzymes to fasting were related to their intracellular distribution, a group of protein-depleted livers was separated by means of fractional centrifugation in isotonic sucrose solution into nuclear fraction, NW, mitochondrial fraction, MW_2, microsomal fraction, P, and soluble proteins, S_2, according to the procedure of Schneider and Hogeboom(10). In Table II relative specific enzyme activity and enzyme content of these fractions are recorded. Locations of maximum enzyme concentration were the mitochondrial fraction for rhodanese, the nuclear and microsomal fractions for arginase, and the microsomal fraction for alkaline phosphatase. The distribution of arginase and rhodanese in protein-depleted livers resembled closely that reported by Ludewig and Chanutin(11) for rat liver in general and also agreed with unpublished data from this laboratory regarding liver tissue from protein-fed rats. The alkaline phosphatase distribution, on the other hand,

§ All rhodanese determinations utilized in Table I were done at pH 7.47 whereas assays on the control group of 76 livers, from which the arginase values of Table I were taken, had been done at pH 7.37. Mean and standard error of the latter group was 217.5 ± 2.87. Unpublished experiments showed that around pH 7.4 a shift of the pH of 0.1 unit results in an activity change of 10%. It appeared advantageous, therefore, to assay hepatic rhodanese at pH 8.8 since there is a broad activity maximum between pH 8.6 and 9.0. The activity at the pH maximum is about 2.5 times that at pH 7.4. The rhodanese assays presented in Table II were obtained at pH 8.8 in 0.1 M 2-amino-2 methyl-1,3 propandiol sulfuric acid buffer.

TABLE II. Intracellular Distribution of Enzymes in Protein-Depleted Rat Liver.*

Enzyme	Nuclei (Nw)	Mitochondria (Mw_2)	Microsomes (P)	Supernatant liquid (S_2)
Rhodanese	.76†(11.9%)§	2.98 (79.4%)	.08‡ (4.3%)	
Arginase	2.21 (23.1%)	.68 (19.2%)	1.53 (42.3%)	.12 (4.1%)
Phosphatase	.66 (8.2%)	.4 (11.4%)	2.00 (51.7%)	.90 (31.8%)
Phosphatase (protein-fed)	.43 (6.7%)	.34 (10.6%)	.92 (23.5%)	2.30 (71.7%)

* Mean values of 2 to 3 experiments.

† Relative specific activity = (units/mg N in fraction)/(units/mg N in homogenate).

‡ Because of the low activity the supernatant from the mitochondrial fraction was not fractionated further.

§ Percentage distribution = [(Units in fraction)/(units in homogenate)] × 100.

differed from that reported by the previous workers in not displaying a concentration maximum in the nuclear fraction. As will be seen from Table II, the distribution differed still more from that found by us in protein-fed livers in which two-thirds of the total phosphatase activity was recovered in the soluble protein fraction. It has been previously pointed out by us(12) that the soluble liver phosphatase is activatable by magnesium ions and not inhibited by 0.01 Molar cyanide, whereas the component associated with particulate matter is highly sensitive to cyanide poisoning, but little activated by magnesium ions. It is the latter component that increases during protein depletion and decreases on fasting.

Discussion. To be designated as a specific response to metabolic stimuli a change in the enzyme content of an organ should be out of proportion to or of a different direction than changes in the content of functionally unrelated enzymes and of the total protein. The fall in the content of the cyanide-sensitive alkaline phosphatase satisfies these criteria. Its possible mechanism and significance have been discussed previously(9,12).

The small increment of the arginase content at the 2 day interval of time has the characteristics of a non-specific response since increments of a similar order were found in the total liver protein and in rhodanese, an enzyme of no known functional relationship and of different location from arginase. The more significant increment of the arginase content between the second and fourth day of fasting, on the other hand, is indicative of a specific response since it was not accompanied by simultaneous increases in rhodanese or total protein content.

It is of interest to recall that a similar sequence of nonspecific and specific responses of arginase as contrasted to consistently nonspecific responses of rhodanese characterized the loss of enzyme activity during extended periods of protein depletion(5). Under both conditions the phase of specific change in arginase content appeared to be the consequence rather than the cause of the altered protein catabolism of the animal. This is in keeping with reports of Folley and Greenbaum(13) and Kochakian and Robertson(14) regarding increases in hepatic arginase following hormonal stimulation of the protein catabolism. The arginase responses, though more than a mere non-specific participation in the protein turnover, are always initiated by simultaneous gains or losses of hepatic protein. They may be designated as excessive formation or excessive deterioration of an enzyme due to a functional overload or nonuse rather than as a strictly adaptive enzymatic response. Their main interest lies in demonstrating that the functional capability of an organ can be altered specifically by nutritional means.

Summary. When protein-depleted rats were subjected to 4 days of fasting, the arginase content of the liver increased by 58% while the alkaline phosphatase content decreased by a similar percentage. A simultaneous smaller increment of the rhodanese content was limited to the first 2 days of fasting. The experiments indicate that the initial increase in arginase activity was due to a nonspecific increase in hepatic protein while the

later rise was a specific response probably related to the increased protein catabolism.

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Effect of Cortisone and other Steroids upon *in vitro* Synthesis of Chondroitin Sulfate. (21164)

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It is known that cortisone inhibits the formation of granulation tissue(1) and that this tissue has a capacity to synthesize chondroitin sulfate(2,3). Layton(4) has further demonstrated that cortisone alcohol inhibits the *in vitro* synthesis of chondroitin sulfate by embryonic and wound tissues. From related physiological information regarding the effects of cortisone in the whole organism(5,6), its action by way of inhibiting chondroitin sulfate formation might well be capable of explaining a variety of observations. While the data presented by Layton(4) provided a reasonable inference that the material being measured was chondroitin sulfate, subsequent studies(7-11) have markedly increased the probability that the measurements are those representing chondroitin sulfate, and confirmation of the inhibition by cortisone alcohol has been provided(9,10).

One of the difficulties inherent in the study of the mode of action of the steroid hormones is the lack of an *in vitro* activity which bears any relation to the action of the hormone in the whole animal. It appeared possible that this inhibition of chondroitin sulfate synthesis by the addition of cortisone alcohol *in vitro* might be sufficiently related to its action in the whole animal, where such inhibition is also observed(12), to provide the *in vitro* tool which is so necessary for this type of problem. However, even the system employed by Layton(4) was somewhat too complex as an initial *in vitro* approach, since it was dependent upon embryonic (granulation) tissue, under conditions essentially comparable to those of tissue culture and required incubation periods of 45 hours. It would be desirable to obtain a somewhat similar system which would employ essentially a tissue slice. This was done by Boström and Mansson(8,9) but a more rapid system was still necessary. This has been achieved as described under methods. Having such a system, which in a period of 5 hours synthesized considerable quantities of chondroitin sulfate, it was found possible to inhibit the chondroitin sulfate synthesis by the *in vitro* addition of cortisone alcohol confirming the results reported by Layton(4) and Boström and coworkers(9,10). However, further studies of this system throw some doubt on the specificity of the effect of the steroid cortisone and on the question of whether this action is really related to the action of cortis-

one in the intact animal.

Methods. The uptake of radioactive sulfur* was used as a measure of chondroitin sulfate synthesis. Two ml of the Tyrodes solution prepared as described by Layton(13) except that carrier sodium sulfate was omitted and the solutions were not autoclaved, plus 0.5 ml of other additions were placed in small flasks or bottles which had been carefully cleaned and soaked in glass distilled water to remove traces of metals. Sulfate was supplied to this solution in the form of carrier-free S^{35}. When steroids were used they were dissolved in chloroform (0.5 mg/ml), the proper aliquot supplied to the flask, and the chloroform removed under vacuum. The Tyrodes solution, etc. were added and the flask shaken in water bath for at least 30 min. before the addition of tissue. This permitted solution of the finely divided steroid in the Tyrodes solution, although the absolute quantity dissolved was not measured.

Rat tissues from normal adult males weighing 250-300 g were employed throughout. The animals were killed by a blow on the head and the tissues removed into ice-cold isotonic KCl. For cartilage, the xiphoid process was employed, trimmed free from bone and cut into slices free-hand. Cartilage slices of approximately 10 mg fresh weight were employed; for tail skin, 100 to 150 mg were used. Incubations with tissue were at 37°C in an atmosphere of 95% O_2 and 5% CO_2. As controls, live tissue was incubated under the same conditions except at 0°C rather than at 37°C. It was felt that this process would permit diffusion, etc., of sulfate ion to take place but should prevent most of the metabolic reactions from proceeding at an appreciable rate.

At the end of the incubation period, the slice was removed from the incubation mixture to a piece of clean blotting paper, the surface dried, and placed in approximately 200 times its weight of 10% trichloracetic acid. This hardened the tissue so that subsequent manipulations were much simpler and the slices did not disintegrate. The slices remained in the trichloracetic acid for 24 hours in the cold. They were then removed, blotted dry, and transferred to fresh trichloracetic acid, where they remained for another 24 hours. They were then extracted in the cold with H_2O and defatted with alcohol and petroleum ether. Analysis of the second trichloracetic extract showed essentially no radioactivity so that at this point it was considered that all of the radioactivity remaining in the slice was not extractable with TCA, and was considered to be chondroitin sulfate. Justification for this procedure is found in previous publications(4,7,8).

Digestion of the cartilage was effected by a micro-Carius combustion. The solutions were made to a known volume and aliquots were taken for sulfur determinations and radioactive measurements. Radioactivity was determined using a thin end-window Geiger-Müller tube with the appropriate corrections made for background, decay and self-absorption. Sulfur was determined by the following procedure. Aliquots of the digestion mixture were made up to 2.5 ml and 0.5 ml of a solution containing 10.0 g $BaCl_2$, 1 ml concentrated HCl and 4.0 g of gum arabic in 100 ml was added. The resulting turbidity was measured in a Beckman Model B spectrophotometer at 620 mμ and compared with known standards of ammonium sulfate precipitated in the same fashion. By this procedure as little as 5 μg of sulfur could be determined.

Initial results with the above system were somewhat low, and means for increasing the incorporation of radioactive sulfur were devised. The Tyrodes solution as prepared contains 1 g per liter of glucose. Increase of this concentration to M/100 (final) seemed to help, but in addition supplementation with

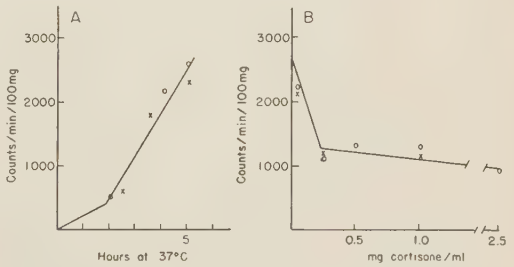

FIG. 1. Uptake of sulfur by rat cartilage *in vitro*. ○ Tissue from adrenalectomized animals. × Tissue from normal animals.

* Radioactive sulfur was obtained on allocation from the Atomic Energy Commission, Oak Ridge, Tenn.

TABLE I. Uptake of Radioactive Sulfur by Cartilage.

Treatment	Wet wt, mg	Total sulfur, μg	Specific activity, c/m/mg S	% uptake of controls†
0°C	15	15.9	10	<.2
	15	5.8	3	
Control	21	10.6	4,113	100
	30	17.2	4,056	
Cortisone	14	16.4	2,998	63
	15	18.1	2,129	
Cortisone acetate	19	*	*	
	13	9.2	2,727	67
Hydrocortisone	21	11.4	11,387	221
	28	11.4	6,658	
Hydrocortisone acetate	25	*	*	
	17	14.9	12,868	730
Desoxycorticosterone	23	16.4	1,385	38
	18	8.2	1,762	
Substance S	22	16.3	4,358	106
	25	*	*	
Cholesterol	17	12.8	5,174	113
	16	16.2	4,049	

* Samples exploded during combustion.
† Avg of the 2 samples.

glutamate (to M/100 final concentration) markedly increased the sulfur incorporation in the case of cartilage. For example, cartilage supplemented with glucose or with glutamate gave 750 to 780 counts/min./100 mg wet weight of tissue, but supplementation with both increased the count to 1870 during a 6-hour incubation period. When both glutamate and glucose were present, but the tissue was incubated at 0° rather than at 37° for the 6-hour period, only 8.7 counts/min./100 mg were incorporated.

Fig. 1A illustrates that after a lag period, the rate of sulfate uptake, and thus presumably chondroitin sulfate synthesis, is a straight line function of time. There is no difference observed between tissues from a normal or from an adrenalectomized animal, the latter being included on the presumption that if cortisone inhibited chondroitin sulfate synthesis, the tissue from adrenalectomized rat (maintained on saline for several weeks), being free from adrenal steroids, should show enhanced chondroitin sulfate synthesis. It is obvious that this is not the case. Nevertheless, Fig. 1B illustrates that sulfate uptake is inhibited by cortisone as reported by Layton (4) and Boström and Odeblad(10).

We therefore chose to regard this system as comparable to that of Layton(4) and with it we confirmed his results. The problem remaining, however, was the relationship of this *in vitro* response to the action of cortisone in the whole intact animal. It had been demonstrated, of course, that there were changes in the skin(5,6) and even in the chondroitin sulfate synthesis(12) on treatment of the whole animal with cortisone, but these observations might be secondary to other changes, and not due to a direct inhibition of chondroitin sulfate synthesis by cortisone. All such changes *in vivo* are observed with cortisone alcohol, cortisone acetate, and other esters thereof, hydrocortisone alcohol, hydrocortisone acetate, but are not observed with desoxycorticosterone or 11-desoxy-17-hydroxycorticosterone (substance S). We therefore reasoned that if the effect observed *in vitro* were related to the *in vivo* observations, the same specificity should be shown.

It can be seen (Table I) that although cortisone and hydrocortisone have identical activity *in vivo,* they have completely opposite effects *in vitro.* Moreover, cortisone and desoxycorticosterone, which are quite unlike *in vivo,* act the same *in vitro.* These con-

tradictory effects observed throw considerable doubt upon the relation of the *in vitro* response observed to the action of these agents in the intact animal. The *in vitro* response, therefore, seems to be related to some property of the steroid which bears no discernible relationship to its physiological action. This conclusion seems to be substantiated by the observation of Jones and Gerarde(14) that cortisone acetate has no effect in essentially the same system used by Layton(4) where the effect of cortisone alcohol was observed.

Summary. An *in vitro* system for the synthesis of acid insoluble sulfate (presumably chondroitin sulfate) has been devised. The synthesis may be influenced (inhibited or stimulated) by the *in vitro* addition of cortical steroids. The influence of the steroids, however, seems to bear no relation to their physiological action. There was no difference in the rate of *in vitro* sulfate synthesis by cartilage from normal or adrenalectomized rats.

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Assay of Influenza Virus Infectivity with Chicken Embryonic Membranes.* (21165)

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(Introduced by Max A. Lauffer.)

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The purpose of this study was to develop an improved assay technic for influenza virus infectivity. The method proposed has several advantages over the titration in chicken embryos in that it requires less labor, less space, and one-twentieth the usual number of eggs. The principal disadvantage is that this new method is not as sensitive. Two animals have proven satisfactory for measuring influenza virus infectivity. These are the mouse(1) and the chicken embryo(2). Bernkopf(3) demonstrated that the virus could be grown on the chorioallantoic membranes of eggs from which the fetus had been removed. There have been references(4-9) to the growth of the virus in cells growing in tissue culture. In view of these findings it was considered likely that small pieces of the chorioallantoic membrane would support influenza virus growth and could, therefore, be used as the host in an assay system for the virus.

Materials and methods. The PR8 strain of influenza virus was used. Stocks had undergone from 17 to 19 egg passages from a pool of preparations A, B, and C described in a paper by Lauffer and Wheatley(10). The individual assay unit consisted of a half-inch test tube containing one ml of nutrient broth, one membrane section, and the appropriate virus inoculum. Membrane sections were prepared by first removing everything except the chorioallantoic membrane from the inside of

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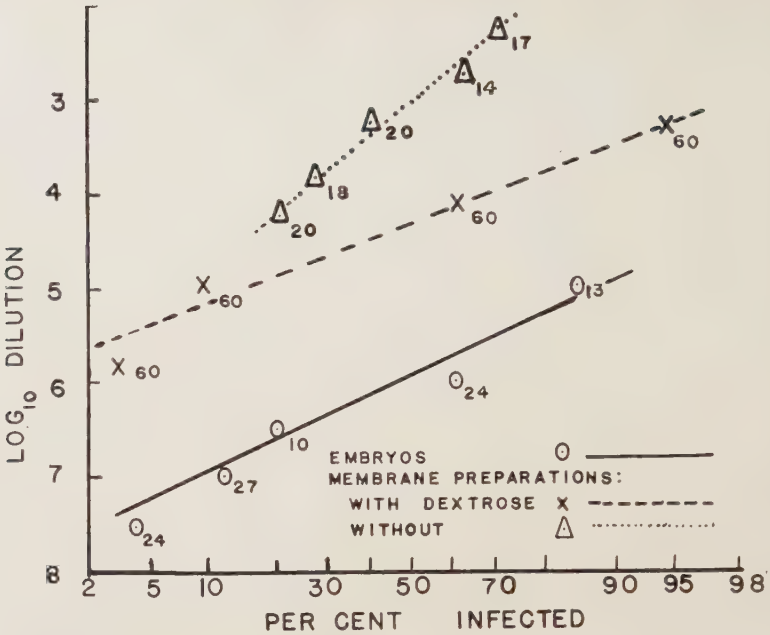

FIG. 1. Comparison of responses with 3 different assay units. Uppermost curve shows distribution of response to PR8 influenza A virus by chicken embryos. The 2 other curves show response of membrane preparations with and without dextrose. Numerals beside the points give number of assay units employed at the given dilution. These points represent the experimental per cent of infection.

9-15-day-old embryonated White Leghorn eggs. The chorioallantoic membranes together with the attached cells and shell membranes were cut into one-quarter inch discs with an ordinary paper punch and were stored from 1-8 days in sterile growth medium at 4°C. The punch and the outside of the shell were sterilized by swabbing with 70% ethyl alcohol. About 16 usable punchings were obtained from an average embryo. The growth medium contained 8 g of nutrient broth, 5 g of sodium chloride, 100,000 units of penicillin, 0.1 g of streptomycin, and, in certain cases, 1 g of dextrose per liter of distilled water. Serial dilutions (usually 10-fold) were made of the virus in broth and the appropriate dilutions were inoculated into the membrane-test tube preparations. The inoculation procedure was to add 0.1 ml of each dilution to each of a given number of these preparations. The inoculated membrane preparations were then incubated at 37°C for 45 hours, or for 112 hours if the broth contained dextrose. The criterion for infection was ability of the incubated broth to agglutinate chicken red blood cells in an 0.8% suspension. The 50% infectious doses were calculated by a modification of the probit method(11). This consists of plotting on probability paper the cumulated percentages of infected membranes against the logarithms of dilution and taking the value of the logarithm of dilution at 50% cumulated response as the endpoint. In certain cases the endpoints were also calculated by an algebraic method(12) equivalent to the method of Reed and Muench(13). Membrane preparation and embryo infectious titers were compared during growth of the virus and during inactivation by urea. The virus was inactivated at 37°C in 1 M urea containing .09 M pH 7 phosphate buffer and 10% broth. During growth, 0.1 ml samples of allantoic fluid were removed at various times from an embryo inoculated with about 10^5 embryo 50% infectious doses of virus The removal was accomplished by inserting a syringe needle through the same hole in the shell that was used for inoculation. Embryo infectious titers were determined in the usual manner (2) using 10- or 11-day White Leghorn embryos.

Results. Two titrations were performed on

TABLE I. Reproducibilities of Endpoints when Membrane Preparations Are Used.

Logarithm of endpoints without dextrose	Logarithm of endpoints with dextrose
3.57	5.43
3.62	6.6
3.40	6.5
3.60	6.5
3.50	5.5
3.03	5.5
4.00	6.9
	6.73
	6.66
	6.42
Avg 3.53 ± .29	6.27 ± .57

the same sample of virus using 14 to 30 assay units per serial 2-fold dilution. The logarithms of the dilutions were plotted as ordinates against the cumulated per cent infections using a probability scale as the abscissa. Straight lines are fitted to this data in Fig. 1. From this figure one can see that the embryos are about 800 times more sensitive than membranes, when the growth media does not contain dextrose.

Reproducibility of these titers was demonstrated by performing 7 independent titrations on the same sample of virus using 5 membrane preparations per dilution and serial 10-fold dilutions. Parallel reproducibility experiments were done in growth media with and without dextrose. A different virus sample was used in each experiment. The results are shown in Table I. The endpoints were calculated by the probit method. The standard error for the probit endpoints is .29 logarithmic units without dextrose and .57 logarithmic units with dextrose in the media. (The differences in standard errors might be attributed to the fact that not all the dilutions were prepared by the same person.) Using 5 animals per serial 10-fold dilution, Lauffer and Miller(1) found a standard error of .260 for mice, and Knight(2) found a standard error of .225 for embryos.

When dextrose is added to the broth, the sensitivity of this assay technic increases about 20-fold. For this reason, although less accuracy is obtained, dextrose was used in subsequent experiments.

When using this technic in growth and inactivation studies, it is desirable to know whether or not the membrane preparations measure the same infectious entity as the embryos. If the same virus preparation is titered by both methods, the embryo infectivity will be different than the membrane preparation infectivity due to a difference in sensitivity of the 2 methods. However, if they both measure the same infectious entity, the ratio of the infectivities, which corresponds to a difference in the logarithm of the infectivity, will be constant during growth or inactivation. The results of experiments performed to test this hypothesis are shown in Tables II and III.

When 4 membrane preparations and 3 embryos per dilution were used, the average endpoint difference was 1.66 ± .24 logarithmic units for growth, and 1.54 ± .25 logarithmic units for inactivation. The ± values are the standard errors of the means. The probability that this difference could be due to errors of random sampling is .76 and, therefore, the difference is considered insignificant. When the data of the 2 tables are pooled, an average difference of 1.60 ± .25 is obtained. In other words, the rate of growth like the rate of inactivation of PR8 influenza A virus infectivity is the same whether it is titered in embryos or in membrane-test tube preparations. These data were also used to plot the response curves of Fig. 1.

Summary. An assay technic has been developed for titrating PR8 influenza A virus infectivity on preparations of chorioallantoic membranes removed from chicken embryos. This technic is simpler and less laborious than the standard assay method involving the use of chicken embryos. It is comparable in ac-

TABLE II. Difference in Growth Values of Virus as Measured in Embryos and Membrane Preparations.

Hr of growth	Logarithm of titer: Embryos	Logarithm of titer: Membrane preparations	Difference
2	3.6	1.3	2.3
4		3.5	
6	5.0	3.6	1.4
8	5.5	4.5	1.0
10	7.5	7.8	1.8
12	9.5	7.0	2.5
24	7.3	9.5	−1.7*
27	8.3	7.3	1.0

* This value was disregarded due to the obvious disagreement with other values.

TABLE III. Difference in Inactivation Values of Virus as Measured in Embryos and Membranes.

Min. of inactivation	Logarithm of titer: Embryos	Logarithm of titer: Membrane preparations	Difference
0	9.5	8	1.5
16	9.2	8	1.2
32	8.2	7.5	.8
48	8.3	5.8	2.5
64	5.5	3.8	1.7

curacy to the embryo technic although appreciably less sensitive. Evidence is submitted to show that both technics measure the same infectious entity.

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A Rapid Pad-Plate Microbiologic Assay for Thymine.* (21166)

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Although several methods(1-4) are available for the microdetermination of thymine, none of these is suitable for the rapid estimation of this material in a variety of biological materials. Williams, Esposito and Pierce(5) have described a microbial plate method for the assay of vit. B_{12}; this makes use of paper pads as originally used in the assay of antibiotics on plates containing a solid assay medium heavily inoculated with microorganisms. As compared with the usual microbiologic methods of assay, this technic affords many advantages, particularly for the assay of large numbers of samples. The present study is concerned with the development of a similar microbial assay method for thymine and thymidine using *Streptococcus faecalis* (ATCC 8043) as the test organism.

Experimental. Materials. Thymine. Material purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio, was further purified by chromatography on Dowex I followed by several recrystallizations from water. *Thymidine* was prepared with the aid of intestinal phosphatase from thymidylic acid; the latter was isolated from calf thymus deoxyribonucleic acid by the method of Hurst, Little and Butler(6). The deoxyriboside was purified by adsorption on Dowex I, hydroxyl form, at pH 11.0; after washing the column thoroughly with 0.01 *N* NaOH followed by water, the thymidine was eluted with 0.04 *M* formic acid. Thymidine was obtained as a dry powder by lyophilization of the formic acid eluant. *Dihydrothymine* was obtained from Dougherty Chemicals, Richmond Hill, N. Y.; *5-methylcytosine* was supplied by G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.; *5-hydroxymethyluracil* and

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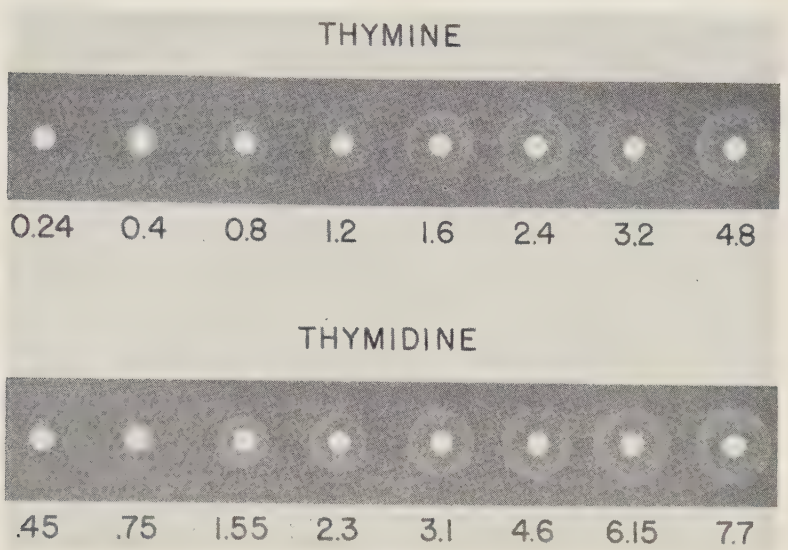

FIG. 1. Growth response of *Streptococcus faccalis* to increasing amounts (μg) of thymine and thymidine.

5-hydroxymethlcytosine were supplied by Sharp and Dohme Division, Merck and Co., West Point, Pa., through the courtesy of C. S. Miller and J. M. Sprague. *Organisms.* Lyophilized cells of *Streptococcus faecalis* (ATCC 8043), used in these studies, were prepared by growing the organisms in 500 ml batches of folic acid assay medium(7) which was supplemented with 1 mg of thymine per liter. After incubation of the inoculated medium for 18-20 hours, the cells were harvested by centrifugation, washed twice with isotonic saline, and finally lyophilized in a lactose suspending medium(8). The dry powder was transferred to small vials and stored in an evacuated desiccator over P_2O_5. The cells of one such preparation have remained viable, when stored in this manner, for a period of more than a year. *Pad Plate Assay.* Solid medium for this assay was prepared as follows: to 125 ml of folic acid assay medium(7) was added lyophilized enzymatic casein hydrolysate (General Biochemicals) (2 mg per ml), aminopterin (5 μg per ml) and agar (1.5%). The agar was dissolved by autoclaving for 10 minutes. After cooling to 50°, 30 mg of 2,3,5-triphenyltetrazolium chloride‡ was added to the medium as a freshly prepared aqueous solution (2 ml). After inoculation of the medium, using the lyophilized organisms suspended in saline (1-2 mg of dry cells were adequate for the inoculation of 125 ml of media), the medium was poured into a suitable tray§ carefully maintained in a horizontal position. The samples to be assayed were placed on small filter paper discs (Schleicher and Schuell No. 740-E, 6.35 mm diameter) with the aid of a Micro-Metric microburette and were allowed to dry at room temperature. These pads, together with pads containing known amounts of thymine or thymidine (which can be prepared previously and stored in small bottles), were placed at suitable intervals on the surface of the agar. The agar was then separated from the edge of the tray with a spatula, and, after covering with plate glass, the tray was placed in a 37° incubator. The zones of growth usually were visible after

‡ It has been found that the addition of 2,3,5-triphenyltetrazolium chloride, which is reduced to an insoluble red compound by the growing cells, improves visibility of areas of faint growth. Blue tetrazolium and neotetrazolium were not as satisfactory as the 2,3,5,-triphenyl derivative.

§ Plexiglass plates (12 x 8 inches) were used. These plates were quite satisfactory if allowed to dry thoroughly under slight pressure for 24 to 48 hours between assays. If this precaution is not observed the plastic becomes hydrated and the plates tend to warp.

5 or 6 hours, however, all recordings were made after an 18-hour period. The diameters of the zones of growth were measured with pointed calipers; the measurements obtained from the standards were used to construct a growth curve from which the amount of thymine on the pads to be assayed could be determined.

Results. The growth response of *S. faecalis* to varying amounts of thymine and thymidine is shown in Fig. 1. It is evident that this method will detect amounts as low as 0.40 and 0.75 μg of thymine and thymidine, respectively. A plot of the diameter of the zones of growth against the amount of compound gives a typical growth response curve.

Specificity. S. faecalis does not respond to the following compounds under the conditions of these experiments (the maximum amount of each compound tested is indicated parenthetically after each compound). Thymidylic acid (75 μg), 5-methylcytosine (6.5 μg), 5-hydroxymethyluracil (28 μg) 5-hydroxymethylcytosine (50 μg) and dihydrothymine (50 μg). In addition to the above mentioned compounds several low molecular weight (di- and tri-) polynucleotides,‖ which were known to contain thymine, but the exact structures of which have not been elucidated, did not support the growth of the test organism. Furthermore, there was no growth of *S. faecalis* when various materials, such as tissue homogenates, known to contain folic acid or its biologically active derivatives, were placed on the plates, indicating that aminopterin had effectively inhibited the utilization of these compounds. It was concluded tentatively that this assay is specific for thymine or thymidine, although it cannot differentiate between the two compounds.

The method has been used in connection with studies of the absorption, excretion and metabolic degradation of thymine and thymidine; the results of these studies will be reported elsewhere. It may be stated, however, that following relatively large oral doses of thymine to rats (up to 400 mg) and to men (up to 6 g) absorption was rapid and nearly complete. Less than one-fourth of the amounts administered appeared in the urine. These findings, together with studies using rat liver slices, have shown that thymine is very rapidly catabolized.

Summary. A rapid microbial (*S. faecalis*) pad-plate assay procedure, specific for thymine and thymidine, is described, in which response to folic acid and its derivatives is prevented by aminopterin. The method is sensitive to thymine in amounts ranging from about 0.4 to 5 μg and to thymidine in amounts of 0.75 to 7.5 μg.

‖ The authors are indebted to Dr. Charles E. Carter for supplying the polynucleotide preparations.

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Effect of Polyvinylpyrrolidone (PVP) on Mouse Liver Function.* (21167)

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Reticulo-endothelial changes occur when large amounts of polyvinylpyrrolidone (PVP) are administered to animals(1-3). Although structural changes due to PVP administration are well established, there is a dearth of information on functional changes. Reinhold *et al.*(4) concluded that PVP seemed to be without ill effects on the liver after they performed a battery of liver function tests on surgical patients given either one or 2 liters of PVP intravenously. No studies were made, however, on non-surgical patients and with the use of larger quantities of PVP. It is the purpose of this paper to report on the use of the bromsulphalein-retention test to determine whether changes in mouse liver function occur after administration of PVP.

Materials and methods. Casals and Olitsky (5) investigated 6 liver function tests applicable to mice and found that retention of bromsulphalein (BSP) was the most sensitive and accurate indicator of liver damage brought about by chemical poisoning, bacterial infection, starvation, and injection of mouse-liver autolysate. In this laboratory their BSP-retention test has been modified as a micro-method which is convenient for use with mice. It allows the use of tail blood rather than heart blood, and requires only 0.03 cc of blood for each test. Some advantages of the micro-method are as follows: the necessity for heart puncture is eliminated; fatalities are nil; daily determinations can be made on the same animal; the BSP level in the blood of a single animal can be determined at several short intervals after injection of the dye to show the changes which occur in the blood BSP level; the small amount of bleeding does not cause a significant change in the condition of the animals due to blood depletion; and anesthesia is unnecessary. The animals used were albino mice of both sexes from the inbred strain BUA maintained at the biological laboratory of Brown University. They were all between 60 and 90 days of age at the time the experiments were begun and were maintained on a diet of Purina Laboratory Chow. The BSP-retention test as routinely used is as follows: 0.125 mg BSP in 0.05 cc physiological saline per g of body weight is injected intraperitoneally. After 20 minutes the mouse is bled from the tail. The blood is drawn into a 0.03 cc micropipette. This blood is mixed with 0.1 cc of a 2% solution of potassium oxalate in saline; thus, the plasma fraction of the blood is diluted approximately 7 times. The mixture is centrifuged at 3000 r.p.m. for 10 minutes and the diluted plasma removed with a capillary pipette. Into each of 2 small test tubes is placed 0.04 cc of this diluted plasma and 0.10 cc of physiological saline. To one tube 0.05 cc of a 10% solution of sodium hydroxide is added to bring out the color of the dye. To the other tube 0.05 cc of a 10% solution of HCl is added to provide a blank. With a capillary pipette these solutions are transferred to very thin cuvettes made from 5 mm glass tubing. An adapter is used for the cuvettes, and readings are made in a Coleman Jr. spectrophotometer at 580 mμ. The values read are compared with a standard curve prepared with known dilutions of BSP and expressed in milligrams per 100 cc of plasma. Values read are multiplied by 7 because of the original dilution of the plasma with oxalate-saline. Standard solutions are treated like the centrifugate in the above procedure. A group of normal mice was used to establish a base line for what might be considered a normal BSP-retention. Another group of mice was injected subcutaneously with a standard dose of 0.1 cc of a 40% solution of CCl_4 in sesame oil which produces a typical central necrosis of the liver. BSP-retention was determined 48 hours later when liver injury was at a maximum (Table I).

* This work comprises portion of thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Brown University.

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TABLE I. Bromsulphalein-Retention in Uninjected Control Mice and in Mice Injected with Carbon Tetrachloride.

Treatment	No. of mice	Bromsulphalein in plasma in mg/100 cc Avg	Range
Controls	35	3.2	2.0- 4.8
Carbon tetrachloride	16	22.6	6.2-41.8

Several groups of mice were injected intravenously, intraperitoneally, or subcutaneously with solutions of PVP which varied in respect to concentration and K-value. The K-value represents the viscosity of the solution from which the average molecular weight of the PVP may be calculated. 1) The K-20 material is a low molecular weight preparation supplied by Schenley Laboratories in powder form. It was used to prepare a 20% solution of PVP in 0.9% NaCl. 2) The K-30 material was supplied in 2 forms by Schenley Laboratories: (A) PVP (MACROSE) was contained in infusion bottles as a sterile solution containing 3.5% by weight of PVP, plus inorganic salts. (B) A powder form from which a 10% solution was prepared in 0.9% NaCl. 3) The K-71 material is a high molecular weight PVP prepared by chemical fractionation by Dr. W. O. Ney of Arnold, Hoffman and Co., Providence, R. I. It was used as a 5% solution in distilled water.

Mice of one group were given intravenous tail vein injections of 0.5 cc of 20% PVP with a K-value of 20 which represents an average molecular weight of approximately 20,000. This volume, given to the mice in a single dose, corresponds approximately to a one-liter infusion in an adult human. The animals of this group were subjected to the BSP-retention test at different times throughout the 3 days following the PVP administration (Table II). Six mice were injected with 0.5 cc of a 20% solution of PVP with a K-value of 20 on alternate days for 15 days. Due to the difficulties encountered in making a long series of tail vein injections, each mouse received 3 or 4 injections intravenously and the remainder intraperitoneally. These animals received a total of 4.0 cc. The BSP-retention test was performed one, 3, 7, 14, and 30 days after the last injection (Table III). Three groups of mice were injected with K-30 PVP, the material which is prepared as a 3.5% solution for human infusions. It has an average molecular weight of approximately 40,000. The mice of one of these groups were injected intravenously with 0.5 cc of a 10% solution given in a single dose. They were then subjected to the BSP-retention test at different times ranging from 0-24 hours, as shown in Table II. The

TABLE II. Bromsulphalein-Retention in Mice Injected Intravenously with a Single Injection (0.5 cc) of Polyvinylpyrrolidone.

K-value	PVP (%)	No. of mice	Hr after inj. of PVP	Bromsulphalein in plasma in mg/100 cc Avg	Range
20	20	6	I*	2.4	1.6-3.2
		6	1	2.2	1.8-2.6
		6	2	3.1	2.0-3.8
		6	4	3.2	1.8-4.6
		6	6	3.5	2.2-4.4
		4	24	3.2	2.8-3.6
		4	72	3.3	2.6-4.2
30	10	6	I	2.9	2.0-4.2
		6	1	3.4	2.8-4.6
		6	2	4.0	2.2-4.8
		4	6	3.2	2.2-4.0
		8	24	3.0	2.0-3.8

* I = Immediately.

TABLE III. Bromsulphalein-Retention in Mice Injected with a Series of 0.5 cc Injections of Polyvinylpyrrolidone.

K-value	PVP (%)	Total inj. (cc)	No. of mice	Days after final inj. of PVP	Bromsulphalein in plasma in mg/100 cc Avg	Range
20	20	4	6	1	2.4	1.8-3.6
			6	3	1.8	1.4-2.4
			6	7	2.9	2.4-3.6
			6	14	3.0	2.4-3.8
			6	30	2.4	1.8-3.6
30	10	5	6	1	2.2	1.2-3.6
			6	3	2.5	1.6-3.6
			6	7	3.2	2.2-4.2
			6	14	3.0	2.2-4.0
			6	28	2.3	1.6-3.8
			4	38	2.6	1.6-4.0
			4	59	2.6	2.0-3.6
30	3.5	10	3	1	2.9	2.6-3.4
			6	17	2.7	1.8-4.0
			6	28	2.9	1.6-4.4
			6	49	2.8	2.0-4.2
71	5	5	5	2	3.3	2.2-4.2
			5	10	3.0	1.8-4.2
			4	30	2.8	1.6-3.8

2 other groups receiving the K-30 PVP preparation were given a series of 0.5 cc intraperitoneal injections totalling 5.0 cc of a 10% solution in one group, and 10.0 cc of a 3.5% solution in the other group. The BSP-retention test was performed on the mice of the first group one, 3, 7, 14, 28, 38, and 59 days after the last injection. The second group was subjected to the test one, 17, 28, and 49 days after the last injection (Table III). Five mice were given a series of 0.5 cc subcutaneous injections totalling 5.0 cc of a 5% solution of PVP with a K-value of 71. The average molecular weight of this highly polymerized material is approximately 125,000. These mice were subjected to the BSP-retention test at 2, 10, and 30 days after the last injection (Table III).

Results. Table I gives the average BSP-retention for untreated controls and for carbon tetrachloride-injected mice, and also shows the range within each group. The group of 35 control animals in which no PVP was administered had an average BSP-retention of 3.2 mg per 100 cc of plasma. The average value for the 16 animals injected with CCl_4 was 22.6 mg per 100 cc of plasma. Since none of the untreated controls showed retention levels above 5 mg and none of the CCl_4 injected animals showed levels below 6 mg, it would seem that readings above 6 mg per 100 cc of plasma could be considered abnormal, while those below 6 mg could be considered within the normal range.

The average and range of the BSP-retention levels after different intervals for the mice given a single intravenous injection of PVP are presented in Table II. The same information for the mice heavily laden with a series of PVP injections is presented in Table III. For all groups of PVP-injected animals the BSP-retention levels are not significantly affected.

Discussion. The early experiments in this investigation were undertaken to demonstrate that the micro-method for BSP-retention was a reliable indicator of liver function and that the presence of circulating PVP did not alter the rate of dye-removal from the blood stream. It was also necessary to determine whether the presence of PVP in the reticulo-endothelial cells would hinder the clearance of BSP from the blood. The micro-method devised for determining BSP levels is a reliable test for poorly functioning livers damaged by CCl_4 (Table I). Experiments in which the dosage and the molecular size of the PVP were varied were designed to detect any dysfunction which might occur in livers which were known to exhibit slight to extensive changes in the reticulo-endothelial cells. The data for all groups of PVP-injected mice show that the BSP-retention levels were not increased. One may conclude from this that the injection of PVP, even in massive doses, does not impair liver function as it is measured by the BSP-retention test.

Summary. A micro-method utilizing blood from tail vessels has been devised for determining the BSP-retention of mice. This method has been shown to be a reliable test for altered function of livers damaged by CCl_4. When applied to mice at varied intervals after PVP administration, the BSP-retention values were invariably within the normal range. Increasing the dosage and the molecular size of the PVP did not increase the dye-retention. The results indicate that PVP, as used in this investigation, does not alter the mechanism of BSP-removal from the blood of normal animals, and it does not cause impaired liver function, either immediate or deferred, as determined by the BSP-retention test.

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Growth-Survival and Sodium Retaining Activity of 9 α-Halo Derivatives of Hydrocortisone.* (21168)

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(Introduced by O. Wintersteiner.)

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Fried and Sabo(1,2) have recently reported a series of 9 α-halo substituted derivatives of hydrocortisone and cortisone possessing unusual glucocorticoid-like activity in the rat. We have examined these compounds for growth and survival as well as for sodium retention in the adrenalectomized rat. The results obtained with 9 α-fluoro, 9 α-chloro-, and 9 α-bromo-hydrocortisone acetate are here reported.

Material and methods. The growth-survival technic of Gaunt *et al.*(3) was used to study the effects of these compounds in maintaining life. Immature male rats, weighing 60 to 70 g, were bilaterally adrenalectomized and immediately injected subcutaneously with an aqueous suspension† of the steroid being tested. The animals used for each test dose were kept as a group in a cage and allowed our stock diet‡ and water *ad libitum*. The cages were inspected daily for dead animals; the rats were weighed twice each week. A survival period of 28 days was used. Animals surviving beyond this period were taken, for purposes of calculation of the data found in Table I, as surviving only 28 days. Data obtained from animals dying within 3 days after adrenalectomy were discarded. The average maximum weight gain, listed in Table I, was determined by summing the maximum gains achieved by each animal in the test group, using a figure of zero for those animals that did not gain or that even lost weight, and dividing by the total number of animals in the group. Daily gain was calculated by dividing the sum of the maximum gains, just described, by the sum of the number of days required by each animal to reach this maximum weight. When the animals either gained no weight or lost weight, their total days of survival were used in obtaining the latter sum. The sodium excretion test used is a modification of the method reported by Dorfman, Potts, and Feil(4). Male rats, weighing 140 to 160 g, were bilaterally adrenalectomized and maintained for 48 hours on our stock diet with tap water containing 1% saline and 5% glucose. This saline-glucose drinking water was then replaced with a 5% glucose solution for an additional 24 hours. Two hours prior to the start of the test all food and water was withdrawn. A minimum of 5 animals per dose level of steroid and 10 animals for untreated controls were used in each assay. Each steroid was tested simultaneously against desoxycorticosterone acetate (DCA) as well as against the untreated controls. Steroids tested for sodium retention were administered by subcutaneous injection of 0.5 ml of the compound dissolved in 20% alcohol. This was followed 1 hour later by subcutaneous injection of 0.5 ml of an aqueous solution containing 35 μg of sodium chloride per g of body weight and 2 μc of Na^{22}. The animals were placed in individual 2-liter beakers containing a raised wire screen for 6 hours. The screen served as a urine-feces separator. After this 6-hour test period, the animals were made to urinate by allowing them to inhale ether and then applying suprapubic pressure. The beakers and screens were thoroughly

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† Aqueous suspensions are prepared by grinding crystalline steroids in mortar and passing through a 200-mesh screen. The finely divided material is suspended in a menstruum consisting of 0.04% Tween 80, 0.7% NaCl, 0.9% benzyl alcohol, and 0.5% carboxymethylcellulose.

‡ The stock diet consists of the following ingredients: yellow corn, 42.5%; linseed meal, 10.5%; casein, 7.5%; alfalfa, 3.0%; skimmed milk powder, 26.0%; yeast, 2.0%; corn oil, 6.3%; cod liver oil, 1.0%; bone ash, 0.7%; and NaCl (fortified with KI), 0.5%.

TABLE I. Growth-Survival of Immature Bilaterally Adrenalectomized Male Rats Receiving Single Injections of Steroids.

Compound	Test dose (μg)	No. of animals	Avg survival time (days)	% surviving 28 days	Avg wt gain Max (g)	Avg wt gain g/day
Aqueous menstruum (controls)	—	105	6.8	2	3.6	.60
E acetate	5000	10	14.1	0	9.5	.87
	2500	10	10.9	10	9.5	1.13
	2000	20	13.4	10	14.6	1.08
	1600	20	10.5	0	3.6	.47
	800	20	9.2	5	9.8	1.40
F "	1600	20	13.5	10	12.4	1.37
	800	9	11.6	0	1.7	.16
DCA	2000	10	16.6	10	27.7	2.40
	1600	10	15.9	0	27.4	2.26
	1000	10	12.0	0	15.8	2.11
	800	39	11.0	5	18.1	2.64
	400	27	10.6	4	11.4	1.93
Bromo F acetate	1600	20	21.2	15	53.0	3.35
	800	19	20.5	31	50.1	3.33
	400	10	9.1	0	12.1	2.14
	200	9	10.1	11	22.3	2.80
Chloro F "	2000	10	28.0	100	72.3	2.96
	1600	19	24.4	74	77.4	3.69
	800	10	17.1	30	51.5	3.75
	400	20	22.9	35	58.3	3.03
	200	9	18.2	11	31.9	2.84
	100	20	13.8	0	27.1	3.00
Fluoro F "	3200	10	8.5	0	.3	.04
	1600	29	12.3	0	6.9	.78
	400	10	8.7	0	5.6	1.24
	100	10	8.7	0	3.1	.49

rinsed with tap water into 250 ml beakers. The combined urine and wash was then evaporated to dryness overnight on a steam bath. Four ml of water were then added to the solids and as uniform a sample as possible was made by scraping the sides and bottom of the beaker with a rubber policeman. Two ml of this suspension were then transferred to one-inch cupped planchets and the amount of radio-activity determined with a scintillation counter.

Results and discussion. From the data in Table I it can be seen that control animals receiving single injections of the aqueous menstruum have an average survival time of 6.8 days. These results are in accord with the results reported by other workers(3). In the groups receiving DCA a survival time of 10.6 to 16.6 days is noted. Very few animals survive the 28-day period. Using DCA as a standard of growth-survival, it can be readily seen in Table I that cortisone acetate (E acetate), hydrocortisone acetate (F acetate), and 9 α-fluoro-hydrocortisone acetate (Fluoro F acetate) elicit much smaller growth responses and somewhat lower survival times. Of these compounds, Fluoro F acetate appears to be the least effective. With 9 α-bromo-hydrocortisone acetate (Bromo F acetate), significant increases were noted in survival time (9.1-21.2 days) and in growth. The data indicate that Bromo F acetate is more active than DCA, since 200 to 400 μg of the bromo compound are about as effective in both growth and survival as 800 to 1000 μg of DCA.

The administration of 9 α-chloro-hydrocortisone acetate (Chloro F acetate) gave the best results in growth survival. All of the animals receiving the 2000 μg dose survived the 28-day period. At the 1600 and 800 μg levels, 74% and 30%, respectively, of the animals survived. When the dose levels were lowered to 100 to 200 μg, the incidence of survival dropped. Even at these dose levels a marked

TABLE II. Comparison of Fluoro F Acetate and DCA in Sodium Retention. (Excretion in Na^{22} expressed as % of controls.)

Dose, μg	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA
2- 3	40	82	72	60	33	36	37	35	41	90
8-10	20	52	61	21	27		37	37	47	
22.5							68	33	44	37

effect on survival time (13.8 to 18.2 days), average maximum gain, and daily gain was noted. From the data in Table I chloro F acetate appears to be 10 to 20 times as effective as DCA in stimulating growth and survival.

The sodium-retaining activity of Bromo F acetate was examined over a range of doses from 0.03 μg to 200 μg. At levels of 2.3 μg and 7 μg, responses of 100% and 80% Na^{22} excretion, respectively, were obtained when compared with controls. At a level of 200 μg the sodium excretion was only 60% that of the controls, indicating that Bromo F acetate is considerably less active than DCA. Application of the F-test in a comparison of dose-response curves between DCA and Bromo F acetate indicates that the curves are not parallel.

Early experiments with Fluoro F acetate in sodium retention showed a significant dose-response curve that was parallel to that obtained with DCA. However, later experiments failed to confirm the earlier observations although analysis of variance(5) of the response showed a highly significant difference between the response for the controls and the treated animals. The data are summarized in Table II and indicate that at dose levels of 2 to 3 μg, Fluoro F acetate is somewhat more effective than DCA in the retention of sodium. At higher dose levels the increased retention of sodium is not always seen with this fluoro derivative.

The possibility that a different mechanism of action exists for the retention of sodium by the fluoro and bromo derivatives than by DCA must be considered.

In contrast, the dose-response curve with Chloro F acetate is parallel to that with DCA (Fig. 1), indicating a similar mode of action for these 2 steroids. The results of 4 independent assays of the chloro derivative are shown in Table III. Using the statistical methods of Bliss(5), the sodium-retaining activity of chloro F acetate, obtained from the

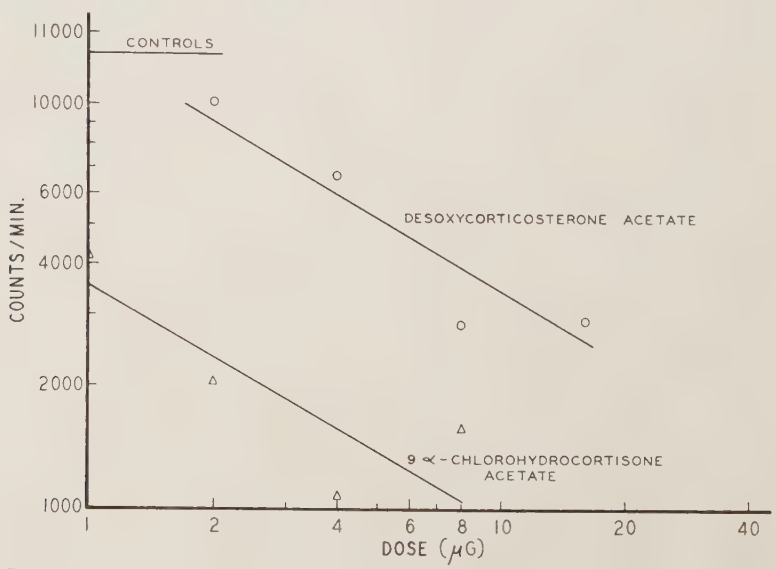

FIG. 1. Comparison of effect of Chloro F acetate and DCA on urinary Na^{22} excretion of adrenalectomized rats.

TABLE III. Assays of Chloro F Acetate for Sodium-Retaining Activity, 4 Experiments.

No. of animals	Potency (DCA = 1)
40	9.5
50	12.3
36	16.9
36	10.2

pooled data listed in Table III, is 10.8 times that of DCA with 95% confidence limits of 6.8 to 17.1. The analysis of variance showed that the departure from parallelism and curvature are not significant. This indicates that the requirements for a valid assay have been met.

The mineralo-corticoid-like activity of Chloro F acetate is of considerable interest in view of the previously reported unusually high glucocorticoid-like action of this compound(1,6). Thus, in the rat this compound is unique in that it manifests both gluco- and mineralo-corticoid-like properties to such a high degree.

It is also of interest to note that growth and survival can be markedly enhanced by a substance, such as Bromo F acetate, which has little sodium-retaining activity. Conversely, a compound with sodium-retaining activity, such as Fluoro F acetate, is not always effective in stimulating growth and survival.

Summary. 1. Growth and survival studies on adrenalectomized immature rats following single injections of aqueous suspensions of steroids indicate that Chloro F acetate is 10 to 20 times and Bromo F acetate 2 to 5 times as active as DCA. E acetate, F acetate, and Fluoro F acetate are less effective than DCA. 2. Sodium retention tests with Na^{22} indicate that Chloro F acetate is 10.8 times as potent as DCA, and that Fluoro F acetate is somewhat more effective and Bromo F acetate considerably less effective than DCA. The dose-response curves suggest that the steroids cause sodium retention by more than one mechanism. 3. The data indicate that, with the steroids studied, there is not always a direct correlation between sodium-retaining activity and growth-survival activity.

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Effects of an Antihyaluronidase Substance and of Hyaluronidase on Growth of Virus-Induced Fibromas. (21169)

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The spreading effect of hyaluronidase has been amply demonstrated by Duran-Reynals (1) and Sprunt(2,3) for India ink, vaccinia virus, fibroma virus, and virus III. We have obtained results similar to theirs with the fibroma virus but have not published these previously since augmentation of the lesion in the rabbit skin brought about by the simultaneous injection of hyaluronidase and virus seemed such an obvious and predictable phenomenon. However, the recent *in vitro* development of an "antihyaluronidase substance" by Hadidian and Pirie(4) stimulated the experiments here described. These indicate that an antihyaluronidase prepared outside of the animal body has an effect exactly the reverse of that of hyaluronidase on the formation of virus-induced fibromas in that it can prevent the formation of the fibroma or markedly diminish its size.

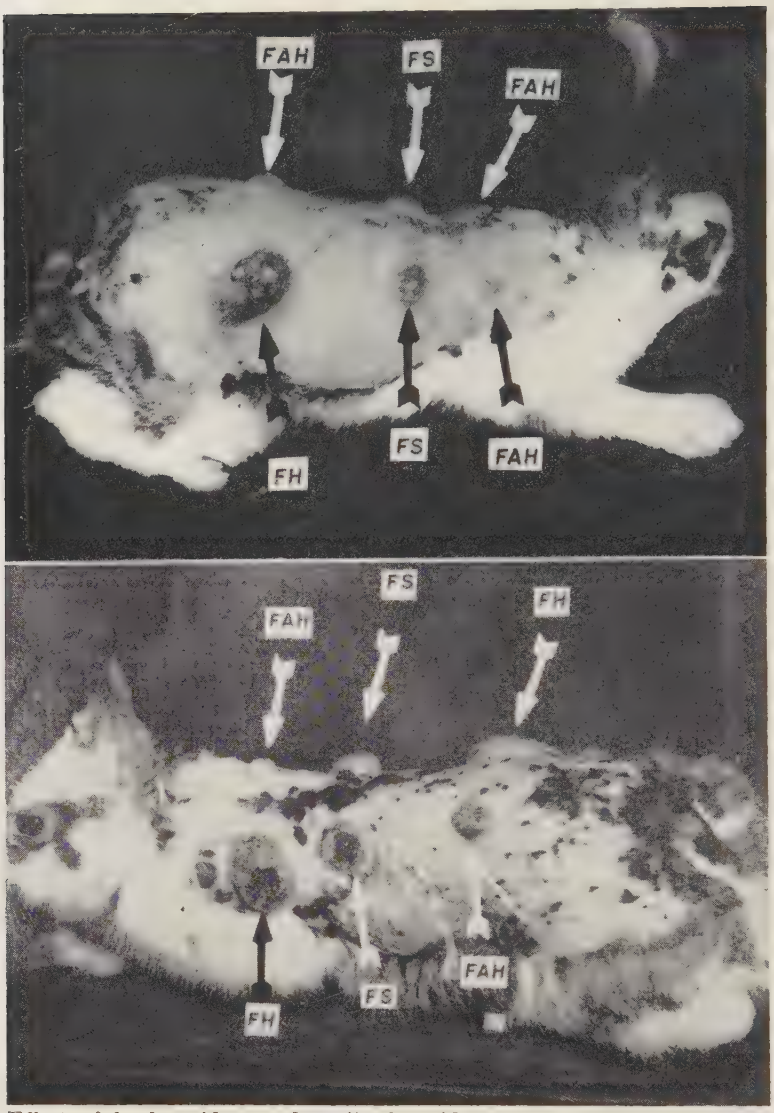

FIG. 1. Effect of hyaluronidase and antihyaluronidase on development of fibroma-induced tumors in the rabbit skin. FH indicates fibroma virus suspension and hyaluronidase injection sites. FS—fibroma virus suspension and saline solution. FAH—fibroma virus suspension and antihyaluronidase. In the rabbit in upper photograph lesions are seen in profile on left side and in lower photograph lesions are seen in profile on right side.

Method and material. The neoplastic or proliferative lesions upon which the hyaluronidase and antihyaluronidase substances acted were produced by the Shope fibroma virus.*

Twelve male rabbits, mixed breeds, weighing approximately 3000 g were used. Six sites of injection were chosen in symmetrical positions on the back of each. One-half cc of a 50% mixture of fibroma virus and antihyaluronidase was injected intradermally into one of the 3 sites on each side. Into another on each side was injected 0.5 cc of a 50% mixture of fibroma virus and hyaluronidase and into the third 0.5 cc of a 50% mixture of fibroma virus and physiological saline. These 3 combinations of material were so placed on the back that the fibroma and antihyaluronidase was at the same level as the fibroma and hyaluronidase. By making this double set of injections there were not only 12 experimental

TABLE I. Effect of Antihyaluronidase on Growth of Virus-Induced Fibromas Compared with That of Hyaluronidase and a Saline Control.

	Fibroma virus + saline	Fibroma virus + antihyaluronidase	Fibroma virus + hyaluronidase
No. of lesions	24	24	24
	cm		
Avg greatest diam.	2.3	1.3	3.5
" least "	2.1	1.2	2.9
" thickness	.5	.2	.5
" vol.	2.4	.3	5.1

animals but each animal was his own control, the similarity of the lesions on the 2 sides of the same animal confirming the validity of the results. The preparations of hyaluronidase and antihyaluronidase were provided by G. D. Searle and Co. of Chicago. The former was their commercial preparation Alidase. The latter was their solution SN752, as described by Hadidian and Pirie(4). Each injection of Alidase consisted of 0.25 cc of the solution or 37.5 turbidity reducing units. Each injection of the antihyaluronidase consisted of 0.25 cc of SN752 or 2.5 mg.

The fibroma virus suspension used for injection was made by grinding portions of the skin tumor produced by a recent serial passage in a mortar and pestle with sterile sand and saline. The same suspension was used in each rabbit.

Results. In every instance the fibroma induced by the virus and a dilution of saline was uniform in size, time of appearance and duration. In every instance the fibroma induced by the virus was inhibited by antihyaluronidase, appeared later, if it appeared at all, and never reached as large a size. In every instance the fibroma induced by the virus is augmented by hyaluronidase, appeared earlier and grew larger (Fig. 1). Since the reactions in the fibroma-saline control sites seemed to reach their height at approximately the 7th day, this time was chosen to record the dimensions of the individual lesions. The tumors were measured in their greatest diameter and in their least diameter, paralleling the skin surface, and in their greatest elevation above it or thickness. The cubic volume was then arrived at by multiplying these 3 figures. These dimensions are listed in Table I. There are other variations in the appearance of the lesions which do not appear in this numerical tabulation. The fibroma-saline lesions were invariable symmetrical, flattened hemispheres. The fibroma-hyaluronidase tumors were not only larger but tended to be asymmetrical, the dependant portions extending downward toward the ventral surface of the animal. There were in addition pseudopodial projections and not uncommonly satellite nodules separated from the main mass by intervening grossly uninvolved skin. These latter never measured more than 2-4 mm in diameter. In the fibroma-antihyaluronidase tumors there was also frequently a marked asymmetry but unlike the hyaluronidase affected lesions this did not seem to be the result of gravity but was quite irregular. Again there were pseudopods and occasionally small satellite masses, but always all of these manifestations were on a minimal scale as compared to the fibroma-hyaluronidase tumors. These differences in the lesion produced by the 3 combinations of injected material were always striking and were constant. There was little deviation among the individual experimental animals from the averages listed in the table.

Discussion. These results as illustrated and as tabulated are so definite that only the mechanism of their accomplishment remains to be considered. Theoretically, there are 2 obvious ways in which the growth of the tumor could be inhibited by the antihyaluronidase. One of these is that the agent that incites growth is no longer active. If the virus is killed by the antihyaluronidase, either in the mixture as prepared before its injection or in the skin site itself, it of course could not pro-

* The fibroma virus used in these experiments was the strain A virus that was originally isolated by Shope(5) in 1932. It was obtained from Doctor Shope in 1938 in the laboratories of the Department of Animal Pathology of the Rockefeller Institute and has been maintained by serial skin passages through the rabbit in the laboratory of the senior author since then. During that time the reaction caused by it has changed in that there is a greater degree of interstitial mucin production and a smaller amount of cellular proliferation. The clinical disease in the rabbit, however, remains unchanged.

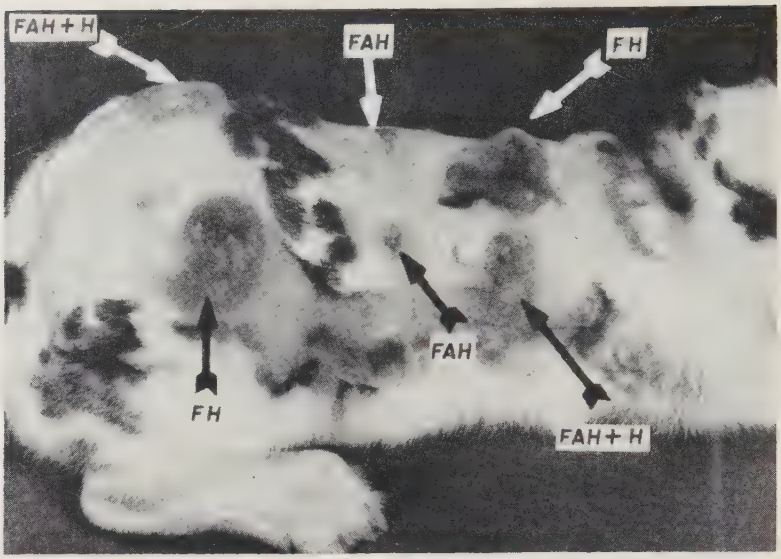

FIG. 2. Effect of a neutralizing amount of hyaluronidase added to fibroma suspension-antihyaluronidase mixture as compared to fibroma virus and antihyaluronidase and fibroma virus and saline. FAH + H indicates the 1st of these combinations, FH the 2nd and FS the 3rd. FAH + H lesions if not entirely equal in size are at least comparable with FH lesions and much larger than FS lesions.

duce tumors. The other possibility is that the power of growth in the reactive cells is inhibited in one way or another.

In order to eliminate the first of these factors, the death or inactivation of the tumor-causing virus, an experiment was set up in duplicate as described above except that at one of the sites of injection on each side a mixture of fibroma virus and antihyaluronidase to which a neutralizing amount of hyaluronidase had been added, was introduced. If the virus was destroyed by the antihyaluronidase no tumor would appear. If the hyaluronidase would counteract or neutralize the antihyaluronidase the tumor would appear and it would be proven that the virus was not killed and the anti-tumor factor was in the action of the antihyaluronidase on the tissues of the host rather than on the inciting virus. The latter proved to be the case. At the sites of injection of the fibroma virus and antihyaluronidase a minimal lesion occurred (Fig. 2). At the sites at which this same mixture with an added equal amount of hyaluronidase was injected, large lesions appeared comparable in size to those at the sites at which the mixture of the fibroma virus and hyaluronidase was injected. This indicates that there is no *in vitro* inactivation of the virus but that the effect of the antihyaluronidase on the lesions produced by the virus is the result of its action on the tissues of the host and not on the inciting virus.

Since in some animals the lesion produced by the virus-antihyaluronidase mixture was minimal or entirely absent it would seem likely that this is not a simple prevention of spread of introduced virus but that it is a prevention of the proliferation of the fibroblast and production by that cell of the large amount of interstitial substance by which this tumor is characterized.

Summary. The action of an antihyaluronidase substance that strikingly inhibits the growth of the tumor produced by the Shope fibroma virus is described. This inhibition is brought about by the action of this material on the cells or ground substance of the host and not by its action on the virus. As previously reported, hyaluronidase increases the size of the tumor-like lesions produced by the Shope fibroma virus in the skin.

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Effect of ACTH upon Artificially Induced Trichinosis in Rats with Special Reference to Eosinophilia. (21170)

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A few reports have recently been published dealing with the therapeutic possibilities of ACTH and Cortisone in the treatment of trichinosis. To study the problem further under controlled experimental conditions, it was deemed advisable to determine the effects of ACTH upon the course of an artificially induced Trichina infection in rats. Such an experiment afforded an opportunity to study the tissue reactions to the parasite in different sites in both treated and untreated animals. The magnitude of the effect of ACTH upon an artificially induced eosinophilia could also be determined(1).

As early as 1904, Opie(2), had described the peripheral blood leucocytosis with both relative and absolute increases of eosinophils and neutrophils in trichinosis in guinea pigs. He also noted myelocytic hyperplasia of the bone marrow and neutrophilic, eosinophilic, and lymphocytic tissue infiltration due to the encysted larvae in muscle(3,4). Luongo(5), *et al.*, reported the use of ACTH for cases of trichinosis in humans. In patients so treated there was observed a diminution in fever, loss of muscle pain, and a striking reduction of other subjective symptoms. Also reported in the same paper were observations on guinea pigs experimentally infected with *Trichina spiralis* and treated with ACTH. A drop in circulating eosinophils and a reduction in fever were reported, but the authors were unable to demonstrate any modification of the inflammatory response.

During our study a paper by Stoner and Godwin(6) appeared in which the effects of ACTH and Cortisone upon the susceptibility to trichinosis in mice was described. They found that these drugs increased the susceptibility of mice to trichinosis, but did not alter the course of the disease or the mortality to a lethal dose of Trichina. In these mice there was no significant change in the degree or type of inflammatory response in treated or untreated animals.

Experimental. Twenty young adult white male rats (Sprague-Dawley strain) weighing on the average from 60 to 80 g were used because of their susceptibility to trichinosis as well as the availability of blood for study. The animals were separated into 4 groups

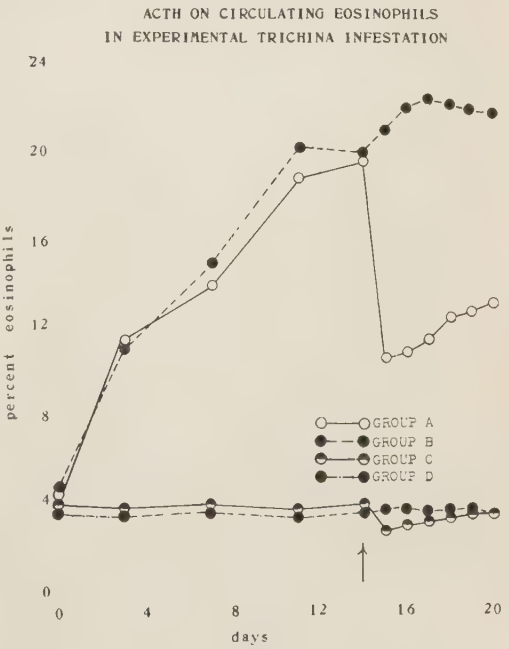

FIG. 1.

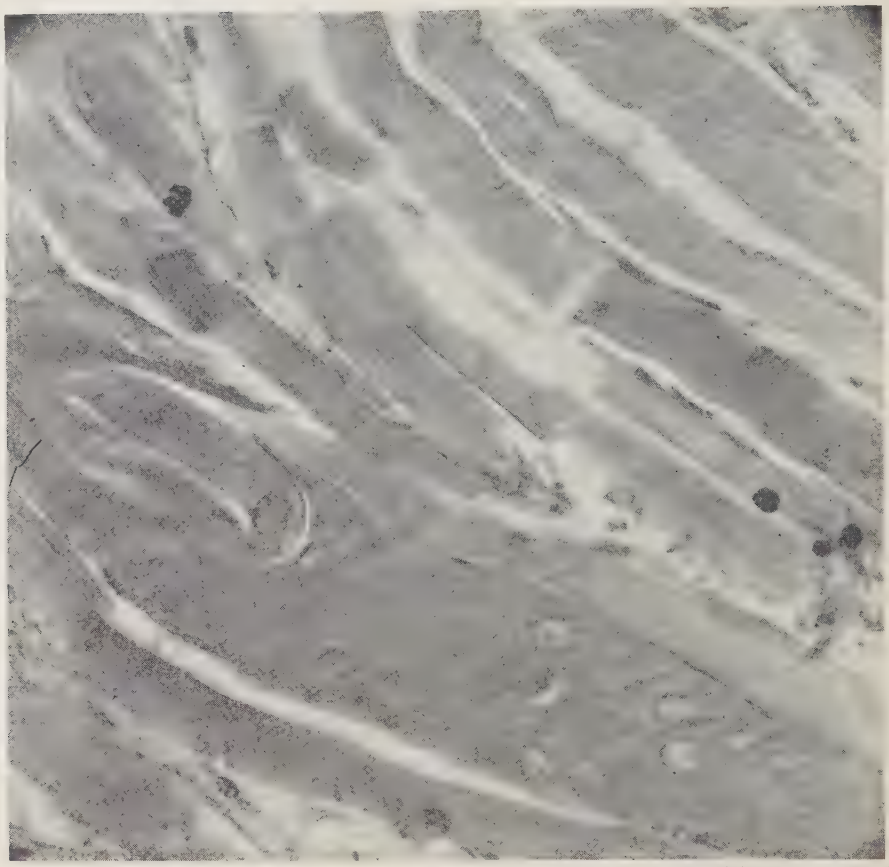

FIG. 2. 600×. Encysted larvae in striated muscle of rats treated with ACTH for 3-4 days.

(A,B,C, and D) with 5 rats in each group. Group A was infected with encysted Trichina larvae and treated with daily subcutaneous injections of ACTH (12.5 mg/day) from the 14th day of infestation until the 20th day. After the 15th day one animal was killed daily. Group B was infected with trichina and treated with daily subcutaneous injections of physiological saline from the 14th day of infestation and sacrificed at similar days as those of group A. The animals of groups C and D were not infected but on days of treatment corresponding to those given above for groups A and B, the rats of group C received ACTH alone and group D physiological saline. The rats were infected in the following manner. Infected rat muscle was fed to the animals following a 24 hour period of starvation. This infected muscle was assayed by counting the larvae in weighed samples and on this basis 3000 larvae were fed to each rat(7).

The course of the disease and the host response was followed by means of total white cell and eosinophil counts made on the 3rd, 7th, 11th, and 14th day of infestation, at which time treatment was instituted and thereafter counts were made every day until the rats were killed. Phloxine stain was used as a diluting fluid for the total eosinophil counts and proved most satisfactory in enhancing the recognition of eosinophils(8).

Histological preparations were made of the following tissues: the diaphragm, intercostal, and femoral muscles; bone marrow; liver; spleen; heart; intestine; and adrenal glands. The tissues were stained with hematoxylin-eosin except for the bone marrow which was stained by the Kingsley method.

Results. Gross observation of the animals

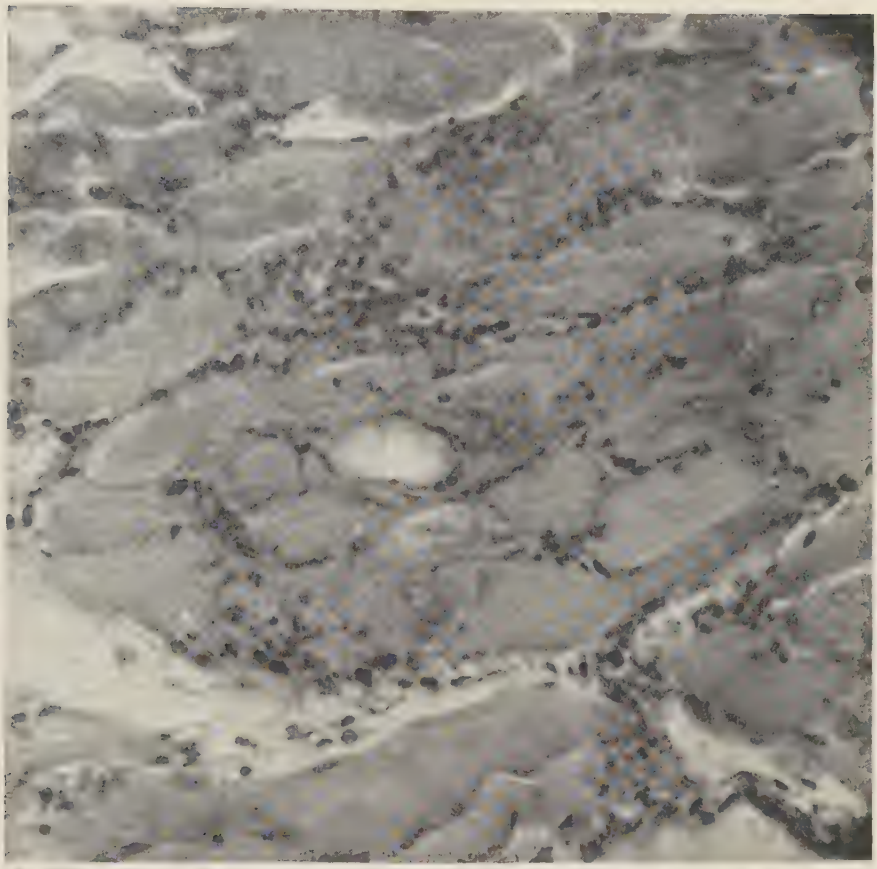

FIG. 3. 600×. Encysted larvae in striated muscle of rats which were untreated (physiological saline).

in the intestinal phase of the disease disclosed marked illness in the infected rats. The signs, although varying in intensity from animal to animal were marked weight loss, diarrhea, anorexia, and greatly reduced physical activity. The gastrointestinal signs decreased during the course of the infestation, but the reduced physical activity was progressive in all infected groups.

In group A the rise in eosinophils was constant and approximated that of group B until treatment was instituted on the fourteenth day (Fig. 1). The daily injections of ACTH had a profound effect on circulating eosinophils for animals of group A. Two distinct phases of the effect of ACTH on circulating eosinophils were demonstrated. The first or sensitive phase is from the 14th to the 17th day of infestation. During this phase there was a precipitous drop in circulating eosinophils from 19.4% to 10.5%. The second or resistant phase is from the 17th to the 20th day. This phase shows the rise in eosinophils even though the remaining animals of this group were still receiving ACTH. Histological sections of striated muscle from this group revealed the typical picture of encysted larvae in muscle of rats treated with ACTH for 3-4 days (Fig. 2). The inflammatory response of animals treated for only 3-4 days has been reduced in comparison to the response in rats treated for 5-7 days, and appears considerably reduced when compared to the response in the untreated animals of group B (Fig. 3). Sections of bone marrow from animals treated with ACTH for 3-4 days showed a myelocytic hyperplasia with great numbers of eosinophils and megakaryocytes

which is somewhat less than that in rats treated for 5-7 days and considerably less than that seen in untreated infected animals. Hyperplasia of the spleen and lymph nodes was noted in both the treated and untreated animals and treatment with ACTH seemed to have no modifying effect.

In group B the rise in eosinophils (Fig. 1) is typical of that found in untreated trichinosis. The injections of physiological saline from the 14th to the 20th day, as might be expected, had no effect on the circulating eosinophils. The normal response of eosinophils to ACTH in a normal animal is illustrated in the curve of group C (Fig. 1). In group D the graph illustrates the normal day to day variation in eosinophils which showed no change with physiological saline injections.

Conclusions. 1. The marked eosinophilia due to an experimental Trichina infection was greatly depressed but not abolished when ACTH was administered for short intervals. 2. The effects of ACTH in animals with artificially induced trichinosis were exhibited by (a) alterations of the inflammatory response to encysted larvae, (b) alterations in the bone marrow, and (c) the changes in the level of circulating eosinophils. 3. Although modification of the host response to Trichina infestation was obtained with ACTH there was no objective evidence that the infected rats benefited as a result of this treatment.

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Steroid-3B-OL Dehydrogenase Activity and Androgen Production in Adrenal and Interstitial-Cell Tumors of Mice.* (21171)

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It has been demonstrated(1) that all normal endocrine tissues which produce non-benzenoid steroid hormones contain an enzyme which will oxidize certain $\triangle^5$-unsaturated steroids with a 3-β alcohol group to the corresponding α,β-unsaturated $\triangle^4$-3 ketone, the latter structure being characteristic of the more active C_{19} and C_{21} compounds formed by these tissues. This enzyme system has not been found in non-endocrine tissues nor in significant quantities in tissues producing only the benzenoid steroids. The present communication reports the presence of this enzyme, 3 β-ol dehydrogenase, in neoplastic tissues arising from the mouse adrenal cortex and testicular interstitial cells, and relates its concentration to the steroid hormone production of these tumors.

Materials and methods. Carcinomas producing sex steroids develop regularly in the adrenal cortices of CE mice several months

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after the animals have been castrated(2). Five such tumors were transplanted into genetically suitable intact recipients in which they grew and formed palpable tumors within 6 to 8 months. These tumors and subsequent transplant generations derived from them were employed in this study. In order to evaluate the production of androgenically active steroids by the grafted tumors, the recipient mice in most instances were castrated when the tumor transplants became palpable, usually from 1 to 3 months prior to the sacrifice of the animals.

Testicular interstitial cell tumors were produced in Bagg albino C mice in three ways: by subcutaneous implantation of a 3 mg fused pellet containing 10% diethylstilbestrol in cholesterol (C ♂ 166298), by rendering the mouse cryptorchid surgically and grafting a pair of ovaries from a young C strain female into the axillary tissue (C ♂ 164041 and C ♂ 166189) or by rendering the mouse cryptorchid surgically and placing it on a semisynthetic diet containing diethylstilbestrol in such an amount that the animal ingested approximately 0.5 γ of the hormone daily (C ♂ 157220). The resultant tumors, regardless of the method of production, were of similar histological pattern, and during the first few transplant generations were "dependent" in that they grew progressively in genetically suitable intact male mice only if these recipient animals were themselves estrogenized. Therefore, with the exception of one experiment, all tumors used in this study were grown in male mice that had been implanted with a fused pellet containing 10% stilbestrol, and in most instances the recipients also were castrated so that the production of androgenically active hormones by the tumors could be evaluated.‡

Androgen stimulation was detected by histological examination of the submaxillary salivary glands and of the seminal vesicles. The degree of stimulation was roughly quantified by weighing the seminal vesicles and adjoining coagulating glands, and in some experiments by weighing the left kidney. In order to simplify the tabulation of data, the degree of androgen stimulation was classified as "minimal" if the seminal vesicle-coagulating gland complex weighed less than 50 mg but the submaxillary glands histologically showed definite evidence of masculinization. The degree of stimulation was considered to be "moderate" in those animals in which the seminal vesicle-coagulating gland complex weight was greater than 50 but less than 150 mg (in such cases the seminal vesicles contained typical white secretion and the submaxillary glands were always of the male type), and as being "strong" when the seminal vesicle-coagulating gland complex weighed in excess of 150 mg.

For estimation of enzyme activity, the tumor transplants were dissected as free as possible from surrounding subcutaneous connective tissue. Histological examination showed that the adrenal cortical carcinomas grew as solidly cellular tumors with very little connective tissue stroma, and grew to fair size with relatively little necrosis of tumor cells. Similarly the testicular tumor transplants contained little supporting connective tissue but most frequently contained cystic spaces filled with sero-sanguinous fluid. The testicular tumors, therefore, were sliced open prior to homogenization in order to release as much of this fluid as possible.

The tumor tissue was then weighed and homogenized in a known volume of the serum-buffer mixture used for incubation. Where high activity was anticipated, the ratio of tissue to total volume was 10 mg per ml; otherwise, the concentration was 15 mg per ml. For each tumor duplicate 125 ml flasks with each of 2 volumes of homogenate (1 ml and 3 ml) were prepared. To each flask, 2 μmols Δ^5-pregnen-3β-ol-20-one were added and the total volume made up to 25 ml with the serum-buffer mixture. This consisted of equal parts of bovine blood serum and Krebs phosphate buffer pH 7.4, to which was added 0.04 M nicotinamide and 0.4 mM diphospho-pyridine nucleotide. The flasks were gassed with oxygen and incubated for 1 hour at 37 degrees. The protein was then denatured by boiling and the mixture was extracted with ethyl ether. Extraction and purification were carried out as

‡ This degree of estrogenization prevents the development of androgen-producing adrenal adenomas which regularly occur in this strain of mouse following castration alone.

TABLE I. Comparison of 3 β-ol Dehydrogenase Activity and Androgen Production in Various Transplant Generations of 5 Adrenal Cortical Carcinomas Arising in Castrate CE Mice. Androgen production by 4 of the tumor lines could not be estimated during the first transplant generation since the recipients had not been castrated prior to sacrifice. Where more than one value is given for enzyme activity, several tumors of the designated transplant generation were analyzed independently. Enzyme activity is expressed as μmols of α, β unsaturated steroid produced per g of tumor per hr of incubation.

	CE ♀ 41	CE ♀ 74	CE ♂ 65	CE ♀ 63	CE ♂ 04
Original donor:					
Androgen stim.	Strong	Moderate	Moderate	Minimal	None
1st transplant:					
Androgen stim.	—	—	"	—	—
Enzyme act.	19.0	9.3-10.0	9.6, 13.5	5.6-10.0	.0
2nd transplant:					
Androgen stim.	Strong		None	Minimal	None
Enzyme act.	27.3		—	7.1	.0, .0
3rd transplant:					
Androgen stim.			None	Minimal	None
Enzyme act.			.0	—	.0-1.7
5th transplant:					
Androgen stim.				Very minimal	None
Enzyme act.				.6-1.4	.4-.6

previously described(3). Conversion of the substrate to an α,β-unsaturated ketone was estimated spectrophotometrically by the height of the absorption peak at 240 mμ.

Results. Adrenal cortical carcinomas. The studies of 3 β-ol dehydrogenase activity in the transplanted adrenal cortical carcinomas are summarized in Table I. Definite activity was evident in 4 of the 5 tumor lines during the first 2 transfer generations, and the concentration of enzyme appeared to parallel roughly the degree to which the secondary male characteristics had been developed as a result of androgenic stimulation. No appreciable concentration of the enzyme was found in the fifth tumor line, and no masculinization of the tumor-bearing mice was evident. Furthermore, one tumor (CE ♂ 65) was moderately masculinizing in the first transplant generation and the enzyme was present in modest concentration whereas in the third transfer generation when the tumor no longer had a masculinizing influence the enzyme was no longer detectable.

Since 3 β-ol dehydrogenase is probably also involved in the synthesis of the active C_{21} steroids, the presence of active glucocorticoids in certain of these tumors is of interest. Tumors of the first transplant generation of CE ♂ 65 and of the second generation of CE ♀ 41, CE ♀ 63, and CE ♂ 04 were pooled for assay, and significant amounts of glucocorticoid activity were detected. When a later transplant generation of CE ♂ 04 tumor which had no appreciable 3 β-ol dehydrogenase activity was assayed alone, no glucocorticoid activity was demonstrable by the Venning procedure(4).

In these adrenal cortical carcinomas it would appear that the concentration of 3 β-ol dehydrogenase roughly parallels the amount of androgen being produced, and that when the tumors contain this enzyme they may also be producing active glucocorticoids. The concentration of the enzyme in the tumors studied, however, was well below that in normal adrenals in CE ♂ mice for on 2 separate assays in which homogenates of pooled whole adrenals were used the amount of steroid oxidized was found to be 150 and 156 μmol/g/hr.

Testicular interstitial cell tumors. All of the testicular interstitial cell tumors assayed were found to exhibit 3 β-ol dehydrogenase activity, Table II. Within any single transfer line of tumor the concentration of enzyme appeared to be moderately constant but seemed to bear no relationship to the production of androgenically active hormone by the individual transplants. The production of androgens by the various transplants differed greatly even when derived from the same primary tumor. This was particularly true in the

TABLE II. Comparison of 3 β-ol Dehydrogenase Activity and Androgen Production in Various Transplant Generations of 4 Testicular Interstitial Cell Tumors Induced in Bagg Albino C Mice. Each enzyme determination was carried out on a single transplanted tumor and comparison is made to the degree of masculinization evident in the individual recipients. All recipients had been implanted with a fused stilbestrol-cholesterol pellet and, where androgen production by the tumor was estimated, the recipients had been castrated.

Original tumor	Transplant generation	Masculinization of recipient	Enzyme activity, μmol/g/hr
C ♂ 164041	1	Strong	27.2
	1	Very minimal	47.4
	2	Minimal	28.6
	3	Strong	29.0
	3	"	33.2
	3	"	29.8
C ♂ 166189	1	None	19.3
	2	Questionable	19.3
	2	None	11.2
	2	Questionable	17.1
	3	Moderate	25.8
	3	"	26.5
	3	Minimal	27.5
C ♂ 166298	3	Strong	10.0
C ♂ 157220 (Line A)	4	Strong	17.2
	8	—	37.6
	8	—	18.4
	9	Strong	18.3
	9	Moderate	15.0
C ♂ 157220 (Line B)	3	None	44.6
	4	"	57.5
	4	"	49.2
	4	"	49.0
	6	—	27.1
	6	—	27.5
C ♂ 157220 (Line C)	3	Strong	7.8
	4	Minimal	22.3

earlier transplant generations studied and in the transfer lines originating from C ♂ 157220 where transfer Line B was maintained distinct from Lines A and C from the time of explantation from the primary tumor. Such variation most probably arose as the result of selection, since most primary testicular interstitial cell tumors vary considerably in histology from area to area, and the histology and function of the explants might be expected to vary depending upon the particular fragment of donor tumor inoculated. Such variation would be expected to decrease as successive transfers are carried out.

Although there appeared to be no obvious correlation between the production of active androgens and the concentration of 3 β-ol dehydrogenase in this group of tumors, it was felt desirable to determine whether variations in hormone production among individual tumors of a single generation of a given transfer line might be accompanied by significant alterations in enzyme concentration. For this study the ninth transplant generation of tumor C ♂ 157220 Line A was employed. The results of this study are summarized in Table III. It can be seen that the concentration of 3 β-ol dehydrogenase was essentially the same whether the tumors were not producing detectable androgen, *e.g.* in intact female animals, or were producing more than the testicular interstitial cells normally do in this strain of mouse, *e.g.* in castrate male animals implanted with 10% stilbestrol pellets and injected daily for the 3 weeks prior to sacrifice with 0.1 mg of horse pituitary gonadotropin (Armour's No. 317-115).§ Even in this latter case the production of androgen per tumor cell is probably much lower than in the normal testicular interstitial cells, the much greater mass accounting for the higher total output.

Accurate comparison of either the androgen production or the concentration of 3 β-ol dehydrogenase in testicular interstitial cell tumors with that in normal interstitial cells is impossible since interstitial cells make up such a small part of the normal testis. Incubation of homogenates of normal mouse testes produce only about 4 μmols of α,β-unsaturated steroid per g of testis per hr of incubation, an amount well below that produced by most of the tumor homogenates studied. Since, however, interstitial cells constitute probably only 1-5% of the weight of the normal testis it seems probable that both androgen production and 3 β-ol dehydrogenase concentration in the normal interstitial cells considerably exceeds that found in the tumors.

Discussion. These studies of 3 β-ol dehydrogenase activity in neoplastic tissues aris-

§ This preparation of horse pituitary gonadotropin contains considerable FSH activity and is approximately 5 times as active with respect to LH as is Armour's sheep pituitary LH preparation No. 227-80. We wish to thank Dr. Sanford L. Steelman of the Armour Laboratories for supplying this hormone preparation.

TABLE III. Comparison of 3 β-ol Dehydrogenase Activity and Androgen Production in Interstitial Cell Tumors of the Ninth Transplant Generation of C ♂ 157220 Line A under Various Experimental Conditions. Enzyme activity was determined on pooled tumors from number of animals indicated in second column, and degree of androgen production was evaluated on the basis of the criteria listed in columns 3, 4 and 5.

Type of recipient	No. of animals	Histology of submax. gl.	Lt. kidney wt (mg)	Seminal ves. wt (mg)	Enzyme activity in tumors, μmol/g/hr
Castrate males	2	♂	204, 187	74, 127	15.0
10% stilb.	3	"	314, 228, 299	205, 249, 264	18.3
Castrate males	2	"	320, 317	285, 316	14.5
10% stilb. Mixed-gonado.	2	"	331, 325	312, 346	15.8
Intact females	3	♀	126, 141, 148	—	14.4
	2	"	161, 168	—	14.6
Castrate males 10% stilb.	10	"	164-205 avg 182	14-49 (Squamous met.)	No tumor
Intact females	11	"	120-165 avg 146	—	" "
Intact males	25	♂	185-304 avg 246	187-323 avg 238	" "

ing from the adrenal cortex and from testicular interstitial cells of the mouse lend themselves to interpretation by recent theories of steroid hormone synthesis in normal tissues. The investigations of Hechter *et al.*(5) indicate that a sequential series of reactions occurs in the adrenal cortex that converts Δ^5-pregnen-3β-ol-20-one to the adrenocortical hormones. The first reaction is that catalyzed by 3 β-ol dehydrogenase resulting in the formation of the α,β-unsaturated Δ^4-3 ketone following which some molecules are oxidized first at C-17 while others are first oxidized at the C-21 position after which 17-hydroxylation cannot take place. They also demonstrated that ACTH acted at some step in synthesis prior to the formation of pregnenolone. Slaunwhite and Samuels(6) have shown that testicular interstitial cells are capable of forming testosterone from pregnenolone by the formation of the α,β-unsaturated Δ^4-3 ketone, followed by hydroxylation at C-17, and then by the splitting off of the side chain at C-17 to yield Δ^4-androstene-3, 17-dione which in turn is reduced to testosterone. Studies of 17 ketosteroid excretion after the administration of various C_{21} steroids indicate that hydroxylation at C-17 is apparently essential for the formation of C_{19} steroids from such compounds(7,8).

The positive correlation between the 3 β-ol dehydrogenase activity and the production of androgens by the adrenal cortical carcinomas studied would indicate that the step catalyzed by this enzyme might well be the limiting one in the synthesis of these steroids by the tumors. According to the postulated sequence just outlined, if this enzyme were the limiting factor for the formation of androgens it should also be limiting in the synthesis of the metabolically active corticoids since the formation of the α,β-unsaturated Δ^4-3 ketone is a step common to both. This would also mean that neither androgens nor corticoids could be formed if 3 β-ol dehydrogenase were absent. This seems to be borne out in the instances where both functions were measured. In the one transplant line of adrenal tumor which lacked the enzyme there was no evidence of androgen production and no glucocorticoid activity could be demonstrated in its extracts: on the other hand 3 of the 4 tumors whose pooled extracts possessed glucocorticoid activity also were producing androgenic compounds and the presence of the enzyme was demonstrated.

The fact that the single adrenal tumor line that did not contain 3 β-ol dehydrogenase had marked estrogenic activity also fits with observations made on normal tissue and in normal animals. Samuels *et al.*(1) did not find 3 β-ol dehydrogenase activity in the follicles

dissected with their membranes from cattle ovaries, even though corpora lutea from the same ovaries were quite active. Heard *et al.* (9) obtained conversion of isotopic cholesterol tó neutral ketonic steroids in the mare, but found no conversion to estrogens. Furthermore when isotopic acetate was administered, the isotope was found in the alicyclic rings of estrone, equilen and equilenin but not in the aromatic portion. Thus, the evidence at present does not support the previous hypothesis that the estrogens are formed by further unsaturation of the neutral steroids.

In the case of the interstitial cell tumors, on the other hand, it is obvious that the step which limits the production of androgenically active compounds by the tumor tissue is not the oxidation of the 3 β alcohol group of ring A. Several tumors that were not producing detectable amounts of androgen exhibited relatively high 3 β-ol dehydrogenase activity while several very masculinizing tumors contained significantly lesser amounts of this enzyme. No tumor was observed, however, that was androgenically active and yet lacked the enzyme. Whether the deficiency in the non-androgen producing tumors occurs at some enzyme-catalyzed step prior to the formation of pregnenolone, resulting in a lack of substrate for 3 β-ol dehydrogenase, or occurs after α,β-unsaturation of the molecule, presumably at some step involved either in 17 hydroxylation or in the splitting of the C-17 side chain, is as yet unknown.

Data obtained in the course of these studies also would seem to bear in a negative manner upon the question of where pituitary LH exerts its principal effect in the chain of events leading to the production of active androgens. Considerable unpublished data, as well as the one study presented here (Table III), indicate that the production of androgens by certain transplanted interstitial cell tumors is, at least in part, under the influence of LH. Alterations in the production of androgens by the one tumor studied, however, was not accompanied by changes in 3 β-ol dehydrogenase activity. This would suggest that LH does not exert its effect primarily by altering the activity of this enzyme. Probably, like ACTH, it exerts its effect at some point rather earlier in the synthesis of the active hormones.

The fact that the 3 β-ol dehydrogenase concentration of the adrenal cortical carcinomas was well below that of normal adrenal cortices is in accord with the general observation that the development of "the malignant change" is usually, if not always, associated with a decrease in specific normal cellular functions resulting from a reduced synthesis of the proteins specialized for such functions. It is obvious in the case of these adrenal tumors that the extent to which the reduction in 3 β-ol dehydrogenase synthesis occurred in association with the development of malignancy varied considerably from tumor to tumor, and that further reductions tended to occur with successive transplantations. Similarly, reductions in 3 β-ol dehydrogenase synthesis probably also occurred during the neoplastic transformation of the testicular interstitial cells, but in this instance greater deficiencies in other enzymes associated with androgen production apparently occurred since in the resultant tumors 3 β-ol dehydrogenase was not the limiting factor in androgen synthesis. Considerable heterogeneity of androgen production was evident in different transplanted tumors originally derived from the same primary tumor. Since this was particularly evident in the early transplant generations, it is most likely explained on the basis of cell selection during transplantation. With further transplantation of any single tumor line, the differences in histological appearance and in androgen production by individual tumors lessened considerably indicating that the fundamental alterations that had occurred were heritable in nature.

Summary. Steroid-3 β-ol dehydrogenase activity was measured in successive transplant generations of experimentally produced neoplasms of the adrenal cortex and testicular interstitial cells of the mouse. Different transplants varied widely in both their masculinizing effect and enzyme activity. In the adrenal tumors the concentration of this enzyme roughly paralleled the masculinizing effect of the transplant, but this was not true in the interstitial cell tumors. Androgenic activity however, was never observed in the absence of measurable 3 β-ol dehydrogenase.

When compared with the sequence of reactions described in normal adrenal cortical and testicular interstitial cells the results are interpreted as indicating that the reaction catalyzed by 3 β-ol dehydrogenase may be the limiting step in androgen synthesis by the adrenal tumors but is not in the case of the testicular interstitial cell tumors.

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Stimulatory Effect of Carbohydrates on Aspartic Acid Deaminase Activity of *Bacterium cadaveris.** (21172)

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Two contrasting effects of glucose have been reported with respect to the aspartic acid deaminase system. The presence of this sugar during growth results in reduction of enzyme activity(1,2), whereas its addition to washed bacterial suspensions results in stimulation of deaminase activity(3). Presumptive evidence for the coenzymatic nature of material resulting from the acid degradation of glucose has been presented recently(4).

The present paper is concerned with the nature of the stimulatory effects of various carbohydrates on the aspartate deaminase system in resting bacterial suspensions, vacuum dried preparations, and cell-free enzyme fractions prepared by sonic vibrations.

Materials and methods. The organism studied was *Bacterium cadaveris* (Gale) cultured at 30°C for 16-18 hours in a medium composed of 1% yeast extract, 1% casitone, and 0.5% K_2HPO_4, adjusted to pH 7. The cells were harvested by centrifugation and washed once with distilled water. Resting bacterial suspensions were prepared by resuspending the washed cells in distilled water to give a cell concentration of 0.3 to 0.5 mg of bacterial nitrogen per ml. Phosphate aging was performed by the method of Lichstein(5) with the cell concentration adjusted to 0.5 to 1.0 mg bacterial nitrogen per ml. Dried cells were prepared by placing washed cell pastes over Drierite *in vacuo* for 24-96 hours; cell-free fractions by treating washed cells in a 9 KC Raytheon sonic oscillator for 10-15 minutes and collecting the supernatant fluid after centrifugation. The deamination experiments were performed in phosphate buffer at 37°C, using adequate controls without added aspartic acid. The reaction was terminated by the addition of 0.2 ml of 25% trichloroacetic acid, the tubes centrifuged and an aliquot of the supernatant fluid removed for ammonia determination by Nesslerization. Color was measured in a Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ).

The carbohydrates tested for stimulating activity were glucose, acid degraded glucose

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TABLE I. Effect of Carbohydrates on Aspartic Acid Deaminase Activity of Phosphate Aged and Vacuum Dried Preparations of *Bacterium cadaveris*.

Additions	μg ammonia-nitrogen produced	
	Phosphate aged resting cells	Vacuum dried preparation
None*	23	28
Biotin, 0.1 μg + adenylic acid, 100 μg	38	46
Yeast extract, 1 mg	46	40
Glucose, 0.5 mg	58	30
" , 5.0 μg	35	28
Acid degraded glucose, 0.5 mg	65	26
Idem, 5.0 μg	33	30
Christman-Williams factor, 0.5 mg	63	27
Idem, 0.5 μg	50	28
None†	2.1	8.8
Biotin, 0.1 μg + adenylic acid, 100 μg	2.3	22.0
Christman-Williams factor, 50 μg	27.0	9.0
Christman-Williams factor, 50 μg + biotin, 0.1 μg + adenylic acid, 100 μg	40.6	21.5

* Reaction time 80 min., 37°C, M/20 phosphate buffer (pH 5). Resting cell suspensions aged in *M* phosphate 45 min. prior to use. Cell conc. = 0.1 mg bacterial nitrogen per ml for resting cell and 1 mg dry wt per ml for dried preparation.

† Conditions as above except resting cell suspensions aged 4 hr prior to use and cell conc. = 0.05 mg bacterial nitrogen per ml.

prepared according to the procedure of Christman and Williams(6), and the Christman-Williams factor prepared and kindly furnished by these workers.

Results. The data given in Table I demonstrate a marked contrast between the phosphate aged resting bacterial suspension and the vacuum dried preparation. All the carbohydrates tested were capable, even in low concentrations, of significant stimulation of the living cell. Since no consistent difference could be found in the activity of the various carbohydrate preparations, it would appear that acid treatment is not required for activity of glucose. This finding is in agreement with the exhaustive studies of Trudinger(3) but does not confirm the observations of Christman and Williams(6). It is pertinent to note that in contrast to the resting cell suspension none of the carbohydrate preparations was capable of stimulating the same system in the vacuum dried cell. That the aspartic acid deaminase was actually resolved may be concluded from the significant stimulations obtained with biotin, adenylic acid, and yeast extract. It is also of interest to note that the additive effect of this factor when combined with biotin and adenylic acid was demonstrable only in the intact cell.

Our findings with the dried preparation are contradictory to those reported by Williams and Christman(4) who demonstrated significant stimulation of this enzyme system by their factor in dried cells. A possible explanation for these differences lies in the method of drying. Williams and Christman employed either a 3-hour drying in a vacuum sublimation apparatus or 16 hours in a vacuum desiccator containing silica gel, whereas we employed more complete drying over a longer period of time. The data given in Table II reveal that until thorough removal

TABLE II. Effect of Drying Time on Carbohydrate Stimulation of Aspartic Acid Deaminase Activity in Vacuum Dried Preparations of *Bacterium cadaveris*.

Additions	μg ammonia-nitrogen produced	
	24 hr drying	72 hr drying
None	14.5	13.1
Glucose, 50 μg	15.4	15.1
Christman-Williams factor, 50 μg	24.6	13.3
Biotin, 0.1 μg + adenylic acid, 100 μg	26.5	24.9

Reaction time 60 min. Other conditions as for Table I.

TABLE III. Effect of Various Carbohydrates on Aspartic Acid Deaminase Activity of Sonic Extracts of *Bacterium cadaveris*.

Additions	μg ammonia-nitrogen produced		
	I	II	III
None	26.1	6.0	25.3
Biotin, 0.1 μg + adenylic acid, 100 μg	35.0		43.0
Yeast extract, 1.0 mg		19.6	36.4
Acid degraded glucose, 250 μg	24.4	6.0	26.0
Christman-Williams factor, 400 μg	29.6	6.1	28.6

Reaction time 60 min., 37°C, M/20 phosphate buffer (pH 7). Cell-free juice contains 0.1 mg nitrogen per ml.

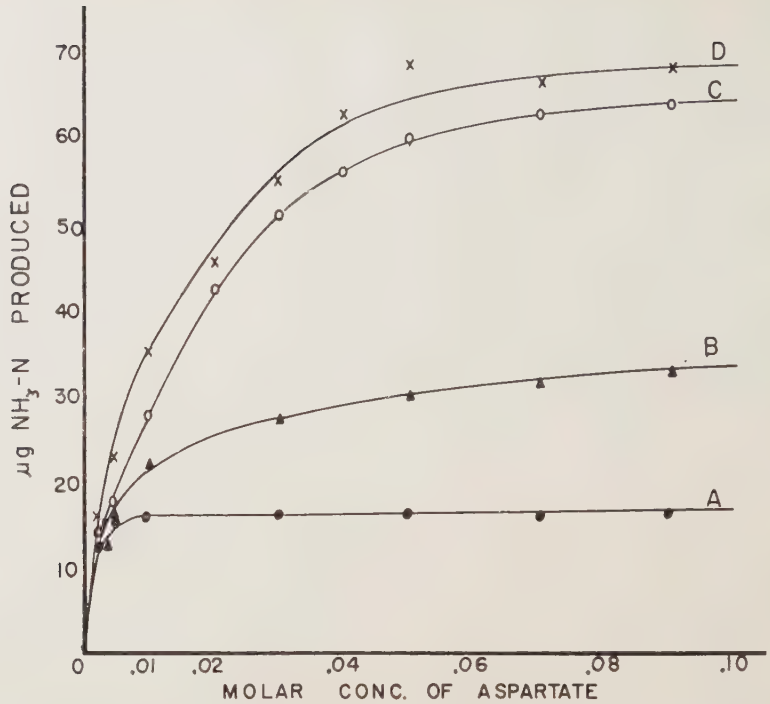

FIG. 1. Effect of substrate concentration on aspartate deaminase activity of *Bacterium cadaveris*. Reaction time 20 min., M/60 phosphate buffer (pH 7). A = living cell = 0.036 mg bacterial nitrogen per ml; B = living cell + 100 μg glucose per ml; C = vacuum dried cell = 1.0 mg dry wt per ml; D = sonic extract = 0.02 mg nitrogen per ml.

of moisture was accomplished the Christman-Williams factor did possess stimulating activity. The negative effects of these carbohydrates on sonic extracts of this organism may be seen by an examination of the results given in Table III, which are in keeping with those obtained with the vacuum dried preparations.

A plot of velocity versus substrate concentration (Fig. 1) reveals that the kinetics of the resting cell could be made to approach those of the sonic extracts and dried preparations if glucose was supplied to the cell. While a definite difference in magnitude was demonstrated between the velocity of the dried preparation, the sonic extract, and the glucose-treated resting cell, all fitted the Lineweaver-Burk equation giving Km values of 11.4 x 10^{-3}, 11.0 x 10^{-3}, and 5.5 x 10^{-3}, respectively, whereas the resting cell without glucose could not be made to fit the equation.

Discussion. It would appear from the data presented that the stimulatory effect of carbohydrates on the aspartic acid deaminase system may be due to an energy requirement for the passage of the amino acid substrate through the cell membrane. However, although the energy requirement for the passage of several amino acids including aspartic acid through the membrane of certain Gram positive bacteria has been demonstrated(7), no information exists with regard to aspartic acid in Gram negative species.

Summary. 1. Glucose, acid degraded glucose and the Christman-Williams factor exhibit significant stimulation of the aspartic acid deaminase system of the living cell. 2. Acid treatment is not required for the stimulatory activity of glucose. 3. None of these carbohydrates is capable of stimulating the thoroughly dried cell or the cell-free sonic extract. 4. The kinetics of the living cell approach those of the cell-free and dried preparation only if glucose is supplied. 5. It appears likely that the stimulatory effect of carbohydrates on this system is an indirect one perhaps concerned with the permeability

of the cell membrane to the amino acid substrate.

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Effect of Dihydrocholesterol and Soy Bean Sterols on Elevated Tissue Cholesterol. (21173)

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Dihydrocholesterol and soy bean sterols have attracted attention because they prevent accumulation of cholesterol in blood and tissues of animals receiving high cholesterol diets. While the exact mechanism of action remains undetermined, it appears that the principal effect of these substances is to diminish absorption of ingested cholesterol(1,2). It seemed important to determine whether mobilization of cholesterol already deposited in tissues can be influenced at all by adding soy bean sterols or dihydrocholesterol to the diet. Rabbits and mice in which tissue cholesterol had been elevated were therefore maintained on diets shown to be effective in preventing accumulation of cholesterol(3), and compared at intervals with animals maintained on control diets.

Experimental. Webster strain female mice weighing 20 g were used. They were fed *ad lib.* a fat-free diet, the composition of which has been covered in detail(3). Their liver cholesterol concentrations were elevated by supplementing this diet with 1% cholesterol and 0.5% cholic acid. After a 2-week period, the animals were divided into 2 groups. One group, the controls, received the unsupplemented diet *ad lib.;* while the other group received the same diet with the addition of 2% dihydrocholesterol* and 0.5% cholic acid, in place of equal percentages of alphacel. This diet has been shown to be efficient in preventing cholesterol accumulation(3) in the mouse. At definite intervals mice were sacrificed from both groups and their liver cholesterol levels determined by a method described previously (3).

In the second experiment, male albino rabbits weighing about 2,000 g were employed. Their plasma cholesterol levels were elevated by feeding *ad lib.*, for a 2-week period, a diet consisting of ground Rockland rabbit chow supplemented with 1% cholesterol and 8% corn oil. The animals were divided into 2 groups. The control group received, *ad lib.*, ground Rockland rabbit chow supplemented with 8% corn oil. The experimental animals received ground Rockland rabbit chow supplemented with 4% soy bean sterols and 8% corn oil. The latter diet when tested proved to be efficient in preventing absorption of dietary cholesterol. At definite intervals blood samples were obtained from both groups and the plasmas analyzed for total cholesterol according to the method of Sperry and Webb (4). The mice in both groups consumed 3.5 ± 0.5 g of food per day; the rabbits 150 ± 25 g per day.

Results. Table I gives the results of the experiments designed to show whether dihydrocholesterol is effective in mobilizing accumulated mouse liver cholesterol. It is apparent that dihydrocholesterol did not cause any acceleration of cholesterol mobilization.

* Generously supplied by the Schering Corporation, Bloomfield, N. J.

TABLE I. Mobilization of Accumulated Mouse Liver Cholesterol by Dihydrocholesterol.

No. of mice	Weeks on diet	Additions to cholesterol-free diet		Total cholesterol remaining in liver	
		Cholic acid	Dihydro-cholesterol	Per organ	Per body wt
		%	%	mg	mg
7	1	—	—	28.1 ± 8.0*	1.3 ± .32*
8	2	—	—	30.6 ± 6.1	1.5 ± .41
8	3	—	—	13.0 ± 3.9	.63 ± .13
7	4	—	—	12.0 ± 4.2	.61 ± .18
7	1	.5	2.5	30.7 ± 7.3	1.6 ± .62
7	2	.5	2.5	31.9 ± 7.1	1.8 ± .61
7	3	.5	2.5	20.1 ± 5.1	1.04 ± .47
7	4	.5	2.5	12.0 ± 4.5	.60 ± .23

* Stand. dev.

Fig. 1 presents the data obtained in the experiments dealing with the rate of fall in elevated total plasma cholesterol in rabbits treated with dietary soy bean sterols and in control animals. Each curve represents the average total cholesterols of 7 animals at various time intervals. Inasmuch as the 2 curves are parallel throughout their entire range, it is clear that in this species the soy bean sterols do not accelerate the rate of decrease of plasma cholesterol levels.

Discussion. The lack of correlation between the results obtained in the prevention of cholesterol accumulation by the use of dietary soy bean sterols or dihydrocholesterol and the mobilization of accumulated tissue cholesterol is of interest. It has been demonstrated that these substances act by preventing the absorption of cholesterol from the intestinal lumen(1,2). Therefore, accumulated cholesterol in these species cannot be mobilized into the intestinal lumen as free cholesterol, or the rate of mobilization would be increased due to the prevention of recycling. It has been shown recently(5) that in the rat accumulated cholesterol is eliminated to a large extent as bile acid. If we assume these results apply in the mouse and rabbit, a possible explanation for our observations is obtained.

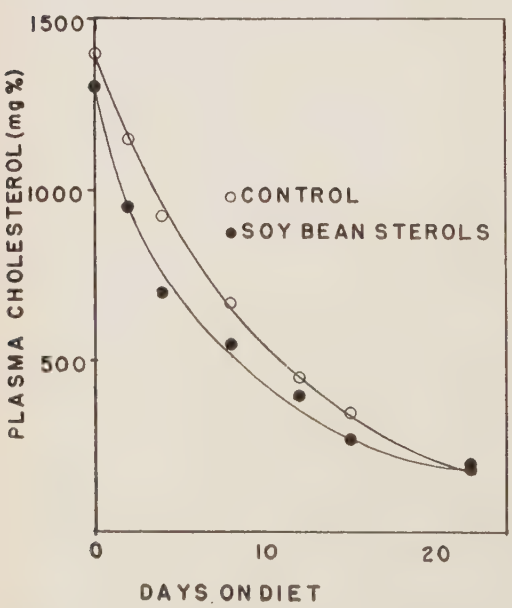

FIG. 1. Rate of decrease of elevated rabbit plasma cholesterol in control animals and animals fed a basal diet supplemented with 4% soy bean sterols.

Summary. 1. Feeding a diet containing dihydrocholesterol to mice with elevated liver cholesterol did not result in an increased rate of liver cholesterol mobilization. 2. Rabbits with elevated blood cholesterol levels were treated with dietary soy bean sterols. The rate of fall of plasma cholesterol was not increased above that observed in a control group.

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Growth of Vaccinia Virus in X-Irradiated Chick Embryo Tissues as Studied in Tissue Culture.* (21174)

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The effects produced by X-rays on innate and acquired immunity of the intact animal have been reviewed(1). In general, the results have been variable, depending on the host-infectious agent system employed and the dosage, time and method of administration of X-radiation. More recent reports (2-5) have shown that prior exposure of the whole animal to X-rays increases its susceptibility to selected viral agents. This effect may be due to an impairment of the antibody response as shown by Dixon *et al.*(6). The use of isolated host cells propagated *in vitro* rather than the intact animal would eliminate many of the immunological factors present in the latter. This paper reports the effects of X-rays on chick embryonic tissue as demonstrated by changes in its ability to support the growth of vaccinia virus in tissue culture. Since this agent in common with other viruses is an obligate intracellular parasite, radiation effects produced in the host cell might be reflected by changes in the amount of viral activity present in the fluid and/or tissue phases of tissue cultures.

Materials and methods. Skin, muscle, and cartilage tissues obtained from 6-14-day-old developing chick embryos were used. The technics of preparing and of incubating roller tube cultures as used in this study followed those described by Feller *et al.*(7). Nutrient fluid containing lactalbumin hydrolysate medium as described by Melnick and Riordan (8) and supplemented with 0.5% chick embryo extract was employed. Penicillin and streptomycin were added to give a final concentration of 50 units/ml and 50 γ/ml, respectively. The strain of vaccinia virus was obtained from Sharp and Dohme, Inc., in the form of calf lymph smallpox vaccine and was carried through 4 serial passages on the chorioallantoic membrane of 12-day chick embryos in our laboratory. Titrations for viral activity were made employing the standard technic(9). Statistical analysis of our titration results showed that the mean of the sample lay within ±20% of the true mean approximately 95% of the time.

Method of radiation. X-radiation was administered as a single massive dose to the tissues in roller tubes or to the chick embryo *in ovo*. The roentgen rays were generated by 2 electrically rectified X-ray apparatuses whose factors were: 1) 100 kv, 4 m.a., filtered through 1 mm Al (HVL 1.53 mm Al), target distance 30 cm delivered at dosage rates of 15.4 and 17.0 roentgens (r) per minute in air as measured through glass (Kimble borosilicate) and egg shell, respectively; 2) 150 kv, 20 m.a., filtered through 0.5 mm Cu plus 1 mm Al (HVL, 0.46 mm Cu), target distance 17 mm delivered at a dosage rate of 102 r per minute in air as measured through the egg shell.

Experimental procedures and results. In the first experiment a comparative study was made of the growth curves of vaccinia virus in tissues which had been exposed to varying amounts of X-rays. Tissue cultures were prepared from 8-day-old chick embryos and incubated in the roller drum for 3 days. At the end of this period, fluids were removed and tissues irradiated employing dosages of 500 r, 1,000 r, and 1,500 r (delivered at 15.4 r per minute). A fourth set of tissue cultures which were not exposed to X-rays served as a control. Fresh nutrient fluid was added to all the tubes and the tissue cultures incubated for 24 hours. At this time, the tissue cultures were inoculated with 2.0 ml of nutrient fluid containing vaccinia virus (3,000 infectious par-

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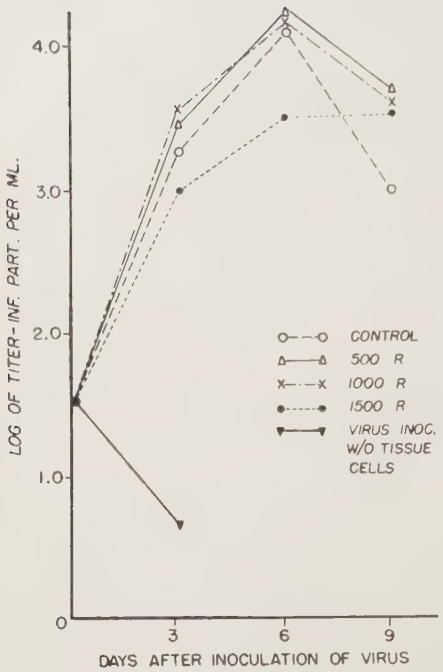

FIG. 1. Growth curves of vaccinia virus in chick embryonic tissue culture exposed to varying dosages of X-radiation.

ticles/ml). As additional controls, uninoculated sets of irradiated and normal tissue cultures were included. Nutrient fluids were replaced at 3-day intervals and the harvested materials titrated in duplicate for viral activity. The results (Fig. 1) showed no significant differences in infectivity titers of the irradiated and normal tissue cultures, with one possible exception. As compared to normal tissue cultures those irradiated with 1,500 r showed a suggestively lower infectivity titer at the end of 6 days.

In the second experiment the effect of serial passage of the virus in normal and irradiated tissue cultures was studied. The virus was carried in parallel through 5 serial passages in normal chick embryo tissues and in similar tissues irradiated with 500 r. The other conditions were similar to those described for the preceding experiment except that tissue cultures were incubated for 5 days, the fluids harvested, diluted 1:50 in fresh nutrient fluid and used as inoculum for the next passage. Infectivity titrations performed on 5th passage material yielded titers of 140,000 and 250,000 infective particles per ml, respectively for irradiated and unirradiated tissues.

In the third experiment the virus was *serially passed* in tissue cultures prepared from chick embryos which had been irradiated with 250 r X-rays *in ovo,* administered at 17.0 r per minute. To this end, 8-day-old embryos were irradiated and reincubated for an additional 24 hours. Tissue cultures were then prepared and incubated for 24 hours. Thereafter, the cultures were inoculated with vaccinia virus and carried through 3 serial passages in parallel irradiated and unirradiated tissue cultures. Conditions of incubation and of serial passage were the same as for Exp. 2. The third passage fluids were titrated and found to contain 850 and 250,000 infective particles per ml, respectively for irradiated and unirradiated tissues.

In the fourth experiment the effects of varying the ages of embryos used and the time of planting tissue cultures after exposure of tissues to irradiation were studied. Embryos 6, 10, and 14 days of age were used. The time intervals between exposure to irradiation and planting of tissue cultures were 0, 24, and 48 hours. Conditions of irradiation as well as planting, incubation and harvesting of fluids were similar to Exp. 3. The inoculum used to infect the tissue cultures had an infectivity titer of 3,000 infective particles per ml. Harvested fluids were titrated and the results are shown in Table I.

In the fifth experiment a study of 2 additional variables, *viz.,* total X-ray dosage (250

TABLE I. Multiplication of Vaccinia Virus in Tissue Cultures Prepared from Chick Embryos of Varying Ages Irradiated (250 r) *In Ovo* and Planted at Stated Time Intervals after Exposure.

Time interval between X-radiation and planting of tissue cultures (hr)	Exp. No.	Age of embryos (days) 6	10	14
0	1	108,000*	18,500	8,000
	2	77,000	15,000	8,000
24	1	28,000	14,800	19,000
	2	34,000	13,200	21,500
48	1	52,500	8,600	11,500
	2	32,500	7,000	6,200
Control (unirradiated tissues)	1	134,000	35,000	9,000
	2	92,000	32,500	11,250

* Infective particles/ml of tissue culture fluid.

TABLE II. Multiplication of Vaccinia Virus in Chick Tissue Cultures Prepared from Embryos Irradiated *In Ovo* with Varying Dosages of X-rays Delivered at Stated Dosage Rates.

Dosages (r)	Exp. No.	Dosage rates (r/min.) 17.0	102.0
250	1	52,000*	42,500
	2	45,000	46,600
500	1	35,500	14,000
	2	48,500	16,000
Unirradiated cultures	1	126,000	
	2	142,000	

* Infective particles/ml of tissue culture fluid.

r, 500 r, and 1,000 r) and rate of administration (17.0 r per minute and 102 r per minute) was carried out. Six-day-old chick embryos were irradiated *in ovo* and allowed to incubate 24 hours. The embryos receiving 1,000 r failed to survive the latter period and were therefore discarded. The remaining embryos were used to prepare tissue cultures. Control cultures were planted employing unirradiated tissues. Both sets of cultures were incubated for 24 hours, inoculated with vaccinia virus and then incubated for 5 days. Fluids were harvested and titrated for viral activity. Results are presented in Table II.

In the sixth experiment the growth curves of the virus in the fluid and tissue phases of irradiated and unirradiated tissue cultures were studied. Six-day embryos were irradiated *in ovo* with 250 r. The chick embryos were incubated 24 hours and then harvested and tissue cultures prepared from normal and irradiated tissues. After an additional 24-hour incubation period both sets were inoculated with virus. Fluids were replaced every 2 days in those cultures which were incubated beyond that period. At intervals beginning with one hour and continuing through 15 days, cultures were harvested. Infected fluids were processed as in previous experiments. Infected tissues were triturated with sterile alundum to which 0.9 ml of Hanks balanced salt solution was added per tissue culture as diluent. The supernatant was removed after centrifugation (2,400 rpm for 20 minutes) and considered to be a 10^{-1} dilution. Titrations for viral activity were performed in duplicate and results used as a basis for Fig. 2.

Discussion. Our experimental results have indicated that the exposure of chick embryo cells propagated in tissue culture to single doses (500-1,500 r) of X-radiation did not affect the ability of these cells to support the growth of vaccinia virus. In contrast, tissue culture cells derived from chick embryos which had been exposed *in ovo* to a smaller dose (250 r) of X-rays 24 hours prior to explantation showed a significant reduction in their ability to support the growth of the virus. These differences in the effects produced by *in ovo* and *in vitro* irradiation suggest that the diminished ability of tissues irradiated *in ovo* to support viral growth was due chiefly to the secondary effects of radiation rather than to primary ones. Further evidence supporting this view is obtained from observation that tissues explanted to tissue culture immediately after exposure to X-radiation *in ovo* were less affected than those irradiated *in ovo* 24 hours prior to explantation. This interval would not enhance the primary effects of radiation but would leave the cell exposed to the influence of secondary radiation effects for a longer time. The significant differences in the mode of action of primary and secondary radiation effects has been reviewed by Lea(10).

The growth patterns of vaccinia virus in chick embryo tissue irradiated *in ovo* and in normal tissue were quite similar. The only

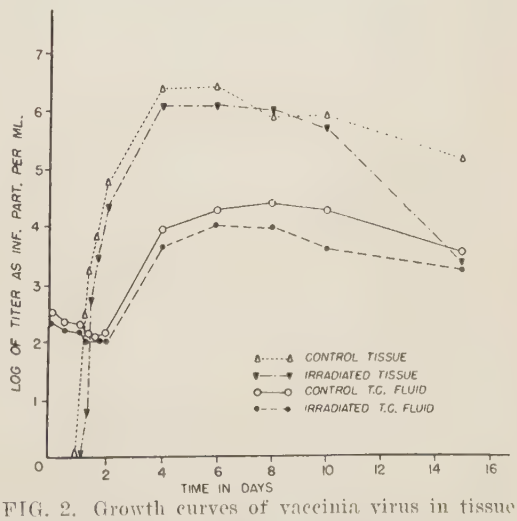

FIG. 2. Growth curves of vaccinia virus in tissue culture prepared from normal and *in ovo* (250 r) X-irradiated chick embryo.

significant difference noted was the markedly lower viral titers obtained in the irradiated tissue cultures. Although direct evidence is lacking it is likely that this diminished ability of cells to support viral multiplication is due to cellular damage resulting from radiation exposure, as has been described in the case of a bacteriophage-X-ray inactivated bacteria system(11). That diminished viral multiplication may be a result of decreased cellular proliferation is suggested by the observation that normal tissue cultures derived from 10-14-day chick embryos yielded lower viral titers than did those derived from younger (6-day) embryos.

The diminished susceptibility of cells irradiated *in ovo* to support viral multiplication is in contrast to reports stating that the radiation of the intact animals results in an increase in susceptibility to viral infection. In another virus-host-cell system utilizing tissue culture technics, we have been unable to overcome the innate resistance of embryonic and adult cells to viral infection by means of exposure to ionizing radiations. Attempts to propagate the MEF1 strain of Type 2 poliomyelitis virus in tissue culture employing mouse embryonic skin and muscle fibroblast-like cells and adult mouse kidney epithelial-like cells previously exposed *in vitro* or *in vivo* to X-rays or beta rays have been unsuccessful.

Summary. The effect of X-radiation of chick embryonic tissue has been studied in regard to its subsequent ability to support the multiplication of vaccinia virus in tissue cultures. Under the stated experimental conditions radiation of the tissue *in vitro* had no significant effect; in contrast, exposure of the embryonic tissue to radiation *in ovo* appeared to diminish its ability to support viral growth in tissue culture. The differences noted were fairly uniform throughout the period studied. Evidence suggesting that the differences noted are due chiefly to secondary radiation effects is discussed.

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A New Count of Allantoic Cells of the 10-Day Chick Embryo. (21175)

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Numerous viruses and some other parasites are often propagated in the cells lining the allantoic cavity of the developing chick embryo. In the case of influenza and some other viruses, many quantitative and kinetic investigations on problems relating to virus reproduction have been carried out with allantoic cells, usually in eggs inoculated at the 10th day of incubation. In order to compute the ratio of adsorbed particles to available cells and the yield of virus particles per cell, it is necessary to know the number of cells lining the allantoic cavity.

This number has been estimated by several indirect methods and by direct count of the nuclei in a preparation, and the various estimates agree fairly well(1). However, recent data obtained in this laboratory indicated that

possibly these estimates were too high(2). The indirect methods employed are valid only if the assumptions on which they are based are also valid. Nuclear counts may have been in error because of the nuclei in other cells as well as the allantoic cells of the chorioallantoic membrane, and the difficulty of distinguishing one from another. In the present investigation the number of cells lining the allantoic cavity was estimated by direct measurement of the mean area of allantoic entodermal cells observed with the phase contrast microscope and by measurement of the area of the allantoic surface. The number of allantoic cells was found to be about 10-fold less than previous estimates.

Materials and methods. Microscopic observations and measurement of cells. The eggs came from a single flock of White Leghorn hens and were incubated for 10 days at 38°C in a commercial egg incubator. The contents of fertile eggs, weighing 54 to 57 g, were removed and the chorioallantoic membrane was rinsed with 0.85% NaCl buffered at pH 7.15 with 0.01 M phosphate buffer (B.S.). With a sharp cork borer (internal diameter 1.26 cm) a circular piece was punched out of the membrane. The piece of membrane with the allantoic surface uppermost was carefully floated from the shell onto a slide and was covered with a coverglass which was sealed to the slide with paraffin. The mean diameter of the piece of membrane was estimated after each count because it tended to shrink gradually to a variable extent. The preparation was examined with the oil immersion objective (97X) of a phase contrast microscope. All 3 layers of the membrane could be visualized, and the uppermost or allantoic layer apparently consisted of a single layer of epithelial cells. The nuclei and cell outlines were clearly seen, as indicated in Fig. 1. Cytoplasmic structures, *e.g.*, mitochondria, were also visualized. A scale drawing of the cell outlines in each field was made on graph paper with the aid of a grid (Howard disc) in the microscope ocular. The grid was calibrated with a stage micrometer employing the objective used in these studies. A grid of 16 squares was used representing a square area of membrane with sides 107 μ in length.

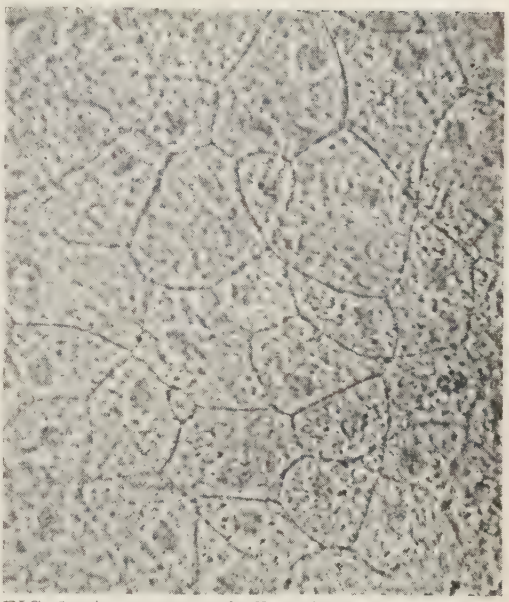

FIG. 1. Appearance of allantoic cells as seen with the phase contrast microscope. Observe clarity of cell outlines. Magnification 505×.

By cutting out the graph area representing the grid and from it the area representing complete cells, and by weighing the 2 parts of the paper, it was possible to calculate the mean area of the cells relative to the grid and hence the mean absolute area of the cells. To obtain the area of the cells in the original membrane, this value was corrected for the amount of shrinkage or stretching of the specimen using the ratio of the square of the internal diameter of the cork borer to the square of the mean diameter of the piece on the slide.

In counting allantoic cells from the amnion-yolk sac area it was not possible to punch out areas of the membrane so as to allow for shrinkage or stretching of the pieces. However, the preparations for microscopy and those for estimating the area of the membrane were prepared by the same operator in as similar a fashion as possible. Therefore, although the area of the cells measured may have been different from that existing before dissection of the embryo, the estimate of the total number of cells present should be correct.

Measurement of the area of the allantoic membrane. The allantoic membrane was removed from the embryonated egg by the following procedure: The pointed end of the egg

TABLE I. Illustrative Protocol of Allantoic Cell Counts on One Piece of Membrane (CA) from a 10-Day Chick Embryo.

Observer*	Diameters of membrane piece	(Mean diameter)^2	No. of complete cells	Wt of traced paper: Whole grid	Wt of traced paper: Complete cells	Mean No. of cells/cm^2†
	cm	cm^2		g	g	$\times 10^5$
1	.94 1.35	1.31	12	.6132	.3658	1.45
2	.9 1.31	1.21	11	.5955	.3094	1.41
3	.9 1.32	1.23	14	.5860	.2701	2.06
4	.92 1.32	1.25	7	.5956	.1950	1.48

* Each observer examined a separate field in the same piece of membrane.

† Calculated = Corrected No. of cells per grid $\times \frac{1}{\text{area of grid}}$ = No. of complete cells $\times \frac{\text{grid wt}}{\text{cell wt}} \times \frac{(\text{mean diameter})^2}{(1.26\ \text{cm})^2} \times \frac{1}{1.14 \times 10^{-4}}$.

was cut off, the egg was immersed in warm buffered saline, and albumin and yolk were drained. The chorioallantoic membrane with the enclosed amnion, embryo, and yolk sac, as well as the remainder of the allantoic membrane, was floated free from the egg shell. The yolk sac then was removed by dissection, and the chorioallantoic membrane was dissected out. It should be emphasized that allantoic membrane in the region of the yolk sac is not fused with this structure. In the region of the amnion, however, the allantoic membrane is partly fused. The various membranes were floated onto a glass surface. The outlines were marked on the glass, from this traced to paper, and the area of the paper was measured by weighing. The area of the membrane attached to the egg shell, *i.e.*, the chorioallantoic membrane, was also measured by an independent method. The outline of the membrane was traced on the shell under the candler. The egg was emptied and the portion of the shell not covered by membrane was cut away. The remaining egg shell was weighed. Eight pieces were carefully punched from the shell with a cork borer of known internal diameter, and these pieces were weighed. From the 2 weights and from the diameter of the cork borer the total area of the shell covered by the chorioallantoic membrane was calculated.

Results. Determination of the area of allantoic cells. The cells enclosed in the grid were drawn independently by 4 different observers. The values for the cell areas were calculated by weight from these drawings; the range of values was about 10% of the mean in one set of 4 drawings and 5% in another set. It was found in later experiments that even when counting cells in different areas individual observers did not count systematically higher or lower than the others. Of approximately 90 intercellular boundaries in one area drawn by 4 observers there was disagreement on the presence of only one such boundary.

Because only about 10 cells were included in each grid drawing on the average and because there was a wide range in cell size, it is possible that the results were biased in that there was more chance of a small cell than a large cell being completely enclosed in the grid. The effect would be to raise slightly the estimate of the total number of allantoic cells present. In one experiment it was found that the effect of increasing the area of the grid 4-fold was to lower the estimate of the mean cell area by 2.5%. This source of error could, therefore, be disregarded.

The allantoic membrane can be described in 2 portions: 1) that fused with the chorion and opposed with it to the shell membrane,

TABLE II. Mean Allantoic Cell Counts on Various Areas of Membrane of 10-Day Eggs.

Area of membrane	Estimated No. of cells per cm² × 10^{-5}			
	Egg 1	Egg 2	Egg 3	Mean
CA near air sac	1.21 (3)*	2.38 (2)	1.58 (4)	1.63 (9)
" " middle	1.90 (3)	1.76 (4)	1.49 (4)	1.71(11)
" " albumen	1.43 (3)	1.57 (3)	2.06 (4)	1.72(10)
Mean	1.51 (9)	1.84 (9)	1.70(12)	1.68(30)

	Egg 4	Egg 5	Egg 6	Egg 7	Egg 8
AYA†	2.60 (3)	2.39 (3)	3.97 (3)	1.60 (3)	4.46 (3)

* Figures in parentheses show No. of counts from which mean is taken.
† Not corrected for shrinkage or stretching of this portion of membrane (*cf.* text).

and 2) that reflected over the yolk sac and amnion and partly fused with the latter. The first portion will be referred to as CA and the second as AYA allantoic membrane. The 2 portions were studied separately.

The CA portion of the membrane was studied first. Table I shows the protocol of counts of allantoic cells made on separate fields of one piece of the CA membrane obtained near the air sac of a 10-day-old egg and illustrates the calculations used. It also indicates that variations occur from field to field which are much greater than variations in drawing cells.

Table II shows an analysis of the results of 30 counts made by 4 observers on 3 areas of CA membrane from each of 3 eggs. It appears that there is no systematic variation in mean allantoic cell area from area to area of the CA allantoic membrane or from egg to egg. Analyses of variance were made which showed that there was no significant difference ($p > .05$) when comparing the variance "within areas" with that "between areas" or that "within eggs" with that "between eggs." On the basis of 27 consecutive counts on 3 eggs it is estimated that the mean number of allantoic cells per cm² is 1.66×10^5 ($\pm$SD 0.082×10^5).

Membranes from twelve 10-day eggs in 4 groups of similar egg weights (one group each: 51-51.5 g and 63-64.5 g; 2 groups: 54-56 g eggs) were counted. Analysis of variance showed no significant relation of egg weight to mean cell area ($p > 0.05$). The mean number of allantoic cells per cm² in this experiment was 1.735×10^5.

The number of allantoic cells per cm² of the AYA portion of the allantoic membrane as shown in Table II was estimated as 3.00×10^5 ($\pm$SD 0.315×10^5) on the basis of 15 counts made on 5 eggs. Analysis of variance showed as with the CA portion of the membrane that the variance "between eggs" was not significantly greater than that "within eggs" ($p > .05$).

Area of the allantoic membrane. The results of a number of measurements appear in Table III. The area of the CA portion of the membrane obtained by weighing of the shell covered by the membrane was consistently higher than that obtained by direct measurement of the membrane. Since the membrane was observed to shrink somewhat while floating in saline, the higher estimate was used for the final calculations. The area of the AYA portion of the membrane was determined by direct measurement only, but the measurements were consistent.

It was found that in eggs incubated from the 4th day without being turned the area of the CA membrane was 31% less than in eggs incubated in the usual fashion. It appears that a previous estimate of the area of the allantoic membrane(3), which agreed with

TABLE III. Area of the Allantoic Membrane.

Egg	Chorionic portion (CA)		Amnion-yolk sac portion (AYA)
	By direct measurement	By wt of egg shell covered	By direct measurement
	cm²	cm²	cm²
A	49.5	58.2	26.4
B	44.2	57.8	30.5
C	49.7	57.8	30.4
Mean*	47.8	57.9	29.1

* Estimated mean total area = 57.9 + 29.1 = 87.0 cm².

TABLE IV. Mean Number of Cells Lining Allantoic Cavity.

Membrane	Mean area of membrane/egg	Mean No. of cells/cm²	Mean No. of cells/egg
Chorionic portion (CA)	57.9 cm² (SD .135)	1.66×10^5 (SD $.082 \times 10^5$)	9.60×10^6 (SD 0.15×10^6)*
Amnion and yolk sac portion (AYA)	29.1 cm² (SD 1.35)	3.00×10^5 (SD $.315 \times 10^5$)	8.73×10^6 (SD 0.97×10^6)*
CA + AYA	87.0 cm²	—	1.83×10^7 (SD 0.1×10^7)

* Approximate estimate ignoring possible covariance.

that reported earlier(1), was in error because in this measurement the area of the yolk sac itself was included. The area of the yolk sac is 35.2 cm² . Subtracting this area from the earlier estimate of 121 cm² gives 85.8 cm² which agrees well with the present estimate which is 87.0 cm².

The number of cells lining the allantoic cavity. From the above data it is possible to estimate the number of cells lining the entire allantoic cavity, and the details of the calculation are set out in Table IV. In the case of the 10-day chick embryo, the total number of cells lining the allantoic cavity is 1.8×10^7. If nearly all the allantoic cells of the CA portion of the membrane were included, the number of allantoic cells in a deembryonated egg would be about 8×10^6.

Discussion. The present value obtained for the number of cells in the allantoic cavity of 10-day chick embryos is about 10-fold lower than that of earlier estimates which have been reviewed recently(1). It is probable that the new value is more nearly correct because the living cells were directly observed in a way that avoided confusion with other membrane layers, and because the present estimate does not rest on unproved assumptions as did some earlier computations. Since much quantitative work, summarized in recent papers(1,2), on the multiplication of influenza virus has been based on the assumption that there are at least 10^8 susceptible cells lining the allantoic cavity, certain interpretations may require reexamination in the light of the present estimate of 1.8×10^7 cells. Recent data from this laboratory(2) indicated that no more than 10^7 virus particles were needed in the inoculum to cause marked alterations in the reproductive process in the allantoic cavity.

Summary. 1. The mean number of allantoic cells per square centimeter of the allantoic membrane in 10-day embryonated chicken eggs has been estimated by direct counting with the phase contrast microscope. 2. In the chorionic (CA) portion of the allantoic membrane there were 1.66×10^5, and in the amnion-yolk sac (AYA) portion 3.00×10^5 allantoic cells per cm². 3. The total area of the allantoic membrane was measured directly. The area of the CA portion was 57.9 cm², that of the AYA portion was 29.1 cm², and that of the entire membrane was 87.0 cm². 4. The number of cells lining the allantoic cavity of the 10-day chick embryo was estimated to be 1.8×10^7.

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Arresting Effect of Heparin upon Experimental Nephrosis in Rats.*† (21176)

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The injection of potent anti-rat kidney serum into rats induces a syndrome which closely resembles the nephrotic state as it occurs in human subjects(1,2). The nephrotic rat thus provides an excellent experimental medium for the study of the mechanism(s) of the abnormal plasma lipid accumulation in this syndrome(3-6). The unique effect of heparin upon the plasma lipids of human patients with nephrosis(7,8) and other hyperlipemic states led to this study of the effect of heparin upon the lipemia of experimental nephrosis in rats.

I. *Effect of Heparin upon Previously Induced Hyperlipemia and Hypercholesteremia of Nephrotic Rats. Methods.* The nephrotic state was induced in a group of 15 male rats (Long-Evans), aged 10 weeks, by intravenous injection of 0.5 ml of rabbit anti-rat kidney serum(1) on each of 2 successive days. The rats were bled from the tail for analysis of plasma total lipids(9) and total cholesterol (10) on the seventh day after the last injection of immune serum. Similar determinations were made in a group of 5 normal control rats. Sodium heparin then was administered subcutaneously, 1 mg twice daily, to 10 of the nephrotic rats and to the 5 normal controls, the other 5 nephrotic animals being kept as untreated controls. Forty-eight hours later all animals again were bled and the plasma total lipids and total cholesterol determined. The turbidity of each plasma sample was determined in galvanometer units proportional to the transmission of light in an Evelyn micro-colorimeter. Distilled water read 100% transmission and total darkness read zero per cent transmission. A 650 mμ light filter was used.

Results. The results are presented in Table I. Seven days after the final injection of immune serum the nephrotic state was well-developed in the 15 rats injected with anti-kidney serum. The subcutaneous administration of heparin to the 10 nephrotic rats led to significant decrease of turbidity of their plasma, accompanied by a marked reduction of the degree of hyperlipemia and a moderate decrease of total plasma cholesterol. In contrast, no change in plasma lipids occurred in the 5 untreated, control, nephrotic rats or in the 5 heparin-treated, normal rats. The administration of heparin for 48 hours to the nephrotic rats, however, did not appear grossly to affect their nephrotic state in other respects.

This demonstrated effect of heparin upon previously developed hyperlipemia and hypercholesteremia of nephrotic rats led to a second experiment, concerned with the effect upon experimental nephrotic hyperlipemia of heparin administered during the induction phase of the nephrotic state.

II. *Effect of Concomitantly Administered Heparin upon the Development of Hypercholesteremia and Hyperlipemia of Nephrotic Rats. Methods.* (a) Fourteen male rats, aged 10 weeks, were injected intravenously with 0.5 ml of rabbit anti-rat kidney serum on each of 2 successive days. Eight rats served as controls, the remaining 6 being daily subcutaneously injected with 2.5 mg of sodium heparin in divided doses. This was administered initially at the same time as the first injection of immune serum and then continued for 7 days. Each rat was bled from the tail on the seventh day for determination of plasma total lipids and total cholesterol. The turbidity of the plasma samples was observed grossly.

(b) In addition, 10 rats were subjected to abdominal laparotomy and cannulation of a lumbar vein with a polyethylene cannula. After closure each rat was placed in a restraining cage and the distal end of the cannula was connected to an individual syringe containing

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TABLE I. Effect of Heparin upon Plasma Lipids of Nephrotic Rats.

	Type of rat					
	Untreated nephrotic		Heparin-treated nephrotic		Heparin-treated normal	
	Before	After†	Before	After	Before	After
No. of rats	5	—	10	—	5	—
Avg wt (g)	140	—	175	—	185	—
Plasma turbidity (units)	21 (3–41)*	18 (5–39)	20 (1–50)	9 (0–24)	0	0
Total plasma lipids (mg/100 cc)	1743 (820–2700)	1657 (1140–2370)	1835 (800–3360)	940 (114–1340)	315 (220–420)	325 (230–425)
Total plasma cholesterol (mg/100 cc)	482 (344–618)	438 (412–468)	469 (242–580)	387 (266–463)	58 (47–74)	47 (38–65)

* Range of values. † 48 hr.

7 ml of isotonic saline. In 5 instances, 5 mg of sodium heparin was added to the infusion fluid. Each syringe was connected with a special continuous intravenous injection apparatus calibrated to deliver 6 ml of infusion fluid in a 24 hour interval. Each of the 10 rats was then injected intravenously with 1.0 ml of potent anti-kidney serum. After 24 hours, each rat was bled for analysis of plasma total lipids and total cholesterol.

(c) In order to confirm the data obtained from this study, a similar experiment was performed in a second series of 25 rats injected with 0.5 ml of anti-kidney serum on each of 2 successive days. Twelve nephrotic rats served as untreated controls, the remaining 13 rats receiving 1 mg of heparin subcutaneously, twice daily, beginning at the same time as the first injection of anti-rat kidney serum. Seven days after the second injection of immune serum the rats were bled from the tail. Heparin injections were stopped and each rat was bled again 4 days later. In each instance plasma total lipids, total cholesterol and degree of turbidity were determined.

Results. The injection of immune serum into the untreated control rats of both series induced ascites, lipemic-turbidity of the plasma and occasionally subcutaneous edema within 48 hours following the last injection. At the time of the initial bleeding (7 days after injection of immune serum), marked ascites and a variable degree of subcutaneous edema could be observed. These findings contrasted sharply with the apparent complete absence of ascites and subcutaneous edema in the heparin-treated rats.

The results of the analyses of plasma lipids in the first series of rats are presented in Table II. Simultaneous administration of heparin subcutaneously during the induction and development of the nephrotic state in 8 rats pre-

TABLE II.
Effect upon Plasma Lipids of Heparin Given during Induction of Nephrosis in Rats.

	Subcutaneous administration of heparin		I.V. administration of heparin	
	Untreated controls	Heparin-treated†	Untreated controls	Heparin-treated‡
No. of rats	8	6	5	5
Avg wt (g)	120	125	130	128
Plasma Turbidity (units)	Present	Absent	Present	Absent
Total lipids (mg/100 cc)	2225 (1540–3400)*	538 (320–814)	496 (305–760)	213 (167–240)
Total cholesterol (mg/100 cc)	428 (264–618)	261 (73–592)	144 (93–210)	69 (62–74)

* Range of values. † 7 days. ‡ 24 hr.

TABLE III. Effect of Heparin Given during Induction of Nephrosis in Rats.

	Heparin-treated rats		Non-heparin-treated rats	
	Day 7*	Day 11	Day 7	Day 11
No. of rats	13	13	12	12
Avg wt (g)	203	—	201	—
Plasma				
Turbidity (units)	0	14 (6–23)†	9.5 (5–17)	21 (8–46)
Total lipids (mg/100 cc)	570 (250–1225)	1975 (910–3040)	1548 (780–2570)	5367 (1300–8870)
Total cholesterol (mg/100 cc)	229 (88–485)	362 (280–505)	373 (230–585)	385 (290–480)

* Heparin stopped on day 7. † Range of values.

vented turbidity of their plasma, in contrast to marked turbidity present in the plasma of the 6 control rats. The heparin-treated group also exhibited a significantly lesser rise of plasma total lipids and total cholesterol. Table II also shows that continuous intravenous administration of heparin appeared completely to suppress the rise of plasma total lipids and cholesterol observed in their paired control rats, 24 hours after injection of immune serum; the values obtained in the heparin-treated group are normal levels for this strain of rats.

The results of the second experiment confirmed those of the first one. It again was observed that simultaneous subcutaneous administration of heparin during the induction phase of the nephrotic state significantly retarded the development of the hypercholesteremia and hyperlipemia of the treated group when compared to the untreated group of rats (Table III). Furthermore, as observed above, the plasma of the heparin-treated rats in each instance was of normal clarity in contrast to variable turbidity of the samples from the untreated rats. However, when heparin administration was stopped in the treated rats for 4 days, their plasma exhibited markedly increased turbidity and a significant rise of total lipids and total cholesterol, although the degree of turbidity and hyperlipemia was much less than that observed at this time in the untreated nephrotic rats.

III. *Effect of heparin upon the clinical picture of experimental nephrosis in rats.* The apparent arresting effect of heparin upon the lipemia and other manifestation of nephrosis observed in the preceding experiments suggested that heparin might be affecting the fundamental pathogenesis of the abnormalities observed in the nephrotic state. This hypothesis was studied in the following experiment.

Material and methods. Ten male rats, aged 8 weeks, were each injected intravenously with 0.5 ml of anti-kidney serum on each of 2 successive days. Five of the rats also received subcutaneous injections of 1 mg of sodium heparin twice daily, the others remaining untreated. Four days later each rat was sacrificed and analysis made of the plasma total lipids and total cholesterol.

Results. From Table IV it can be noted that the heparin-treated rats injected with immune serum did not exhibit gain of weight, ascites, subcutaneous edema, or turbidity of their plasma. A moderate rise of cholesterol and of total lipids was found in these rats. In contrast, the control rats which similarly were injected with immune serum but which did *not* receive heparin exhibited marked ascites, a variable degree of subcutaneous edema and a consequent average gain of weight of 39 g, and marked lipemic-turbidity of their plasma. A considerably greater rise of plasma lipids and of cholesterol also was observed in these animals.

Discussion. The present data indicate that heparin apparently is capable of profoundly influencing the nephrotic state in rats. It was found initially that heparin induced a significant reduction of plasma lipids and lipemic-turbidity in rats previously made nephrotic (Table I). This response was anticipated in

TABLE IV. Effect of Heparin on Lipids and Other Signs of Nephrosis in Rats.

	Heparin-treated rats	Non-heparin-treated rats
No. of rats	5	5
Avg wt (g)		
Day 0	139	142
" 7	143	181
Ascites	Absent	Present
Subcut. edema	"	"
Plasma		
Turbidity (units)	"	"
Total lipids (mg/100 cc)	376 (335–460)*	1333 (520–2570)
Total cholesterol (mg/100 cc)	141 (121–191)	338 (226–487)

* Range of values.

view of the findings by others that heparin similarly lowered the plasma lipids of nephrotic humans(7,8). It seems likely that the changes induced by heparin in the physicochemical state of the plasma lipids(11,12) facilitates the removal of excess lipids from the plasma by the liver, with consequent reduction of the degree of hyperlipemia and hypercholesteremia.

Heparin appeared to exert a greater effect upon plasma lipids when administered during the induction and development phase of the nephrotic state in rats. Thus, clear plasma and markedly lower levels of plasma lipids were observed in the animals treated with heparin following injection of anti-kidney serum, in contrast to untreated rats also injected with anti-kidney serum (Table II). This inhibitory effect was striking when heparin was administered by continuous intravenous infusion following injection of immune serum. On the other hand, the suppressive effect of heparin upon retention of excess lipids in the plasma of the nephrotic rat apparently was of a temporary nature, as cessation of heparin therapy was followed by progressive rise of hyperlipemia, hypercholesteremia, and the appearance of lipemic-turbidity of the plasma (Table III).

Although heparin treatment consistently reduced lipid levels in the rat with previously induced nephrosis, it did not appear grossly to affect other aspects of the nephrotic state.

However, administration of heparin during the induction phase of the nephrotic state appeared to exert a profound arresting effect upon other aspects of the nephrotic syndrome. Administration of heparin during the development phase completely or almost completely prevented such other "clinical" manifestations of the nephrotic state as subcutaneous edema, ascites, and weight gain (Table IV). In Fig. 1 can be seen the typical appearance of the edematous nephrotic rat 7 days after injection of immune serum, initially similar in weight and appearance to the heparin-treated rat in which edema and ascites were not observed at this time. However, when heparin was stopped, there was a delayed appearance of moderate ascites and occasionally subcutaneous edema, in association with a rise of plasma lipids. In subsequent studies we have observed that much larger doses of heparin are required to suppress the "clinical" signs and the hyperlipemia induced by injection of exceedingly high potency anti-kidney serum, smaller doses of heparin sufficing when less potent immune serum is used.

It seems likely that the degree of nephrotic lipemia is proportional to the severity of the underlying abnormality, and therefore it appears unlikely, that the apparent suppressive effect of heparin upon the development of the nephrotic state is merely secondary to its effect upon the plasma lipids. At present it seems plausible that heparin has some effect upon the causal mechanism whereby nephrosis is induced in rats by injection of rabbit anti-rat kidney serum. Ehrich and his associates (2) extensively studied the pathological

FIG. 1. Two rats injected with anti-kidney serum 7 days previously: Left, treated with heparin daily for 7 days. Right, untreated control.

changes in the kidneys of nephrotic rats. They emphasized the close resemblance to the morphological changes observed in the kidneys of humans with lipid nephrosis and suggested that the immunologic reaction induced by injection of anti-rat kidney serum is of the nature of a Shwartzman reaction. Thus, heparin might act by preventing such a Shwartzman reaction since it has been shown that heparin is fully capable of preventing both the local and the generalized Shwartzman reaction(14,15). Heparin also might influence the reduction of serum complement induced by injection of anti-kidney serum, shown to occur in rats which develop nephrosis following injection of immune serum(13). Finally, heparin might act in a different manner by suppressing thrombosis of the capillaries and alterations of the basement membranes of the glomeruli which follow injection of anti-kidney serum(2).

Our results agree in part with the report of Kleinerman(16) that heparin was found to prevent or markedly suppress "clinical" signs and pathological renal lesions induced in rabbits following injection of (duck) anti-rabbit kidney serum. Ehrich and associates(2) have pointed out that although anti-kidney disease in rabbits more closely resembles glomerular nephritis in humans with an immunologic reaction likened to the Arthus phenomenon, the response to injected anti-kidney serum in rats and in rabbits appears to be a different immunologic reaction to the same factor(2).

Summary. Heparin administration decreases the hyperlipemia and hypercholesteremia of rats with established nephrosis, induced by injection of rabbit anti-rat kidney serum. Heparinization during the *induction* phase of the nephrotic state appears to suppress or prevent ascites and edema, and to reduce markedly the degree of lipemia, hypercholesteremia and lipemic-turbidity of the plasma. Possible mechanisms of this arresting effect of heparin are discussed briefly and it is suggested that heparin suppresses the causal mechanism whereby nephrosis is induced in rats by injection of anti-kidney serum.

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Selective Susceptibility of Ateles Monkeys to Infection with Type I Poliomyelitis and Col SK Virus.* (21177)

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It is well known that various species of monkeys differ in their susceptibility to infection with poliomyelitis virus; generally speaking, Old World monkeys are considered more susceptible than those of the Western Hemisphere. Among the New World monkeys the genera *Cebus, Alouatta,* and *Ateles* have been investigated to some extent. Thus, ringtail monkeys (*Cebus capucina*) from Central or South America have been variously reported as being refractory(1-3), or susceptible (4,5) while Panamian howler monkeys (*Alouatta palliata*) proved susceptible in the experience of one investigator(5). Negative results were obtained by 2 workers(5,6) with spider monkeys (*Ateles fusciceps robustus* or *Ateles ater*). In many of these experiments comparatively small numbers of animals were studied. The significance of the results may, therefore, be influenced by host factors connected with the geographic origin or race of the animal as well as by the choice of virus strain used. In view of these uncertainties, the efforts of 2 laboratories were combined to reinvestigate the status of susceptibility of Ateles monkeys to infection with the 3 serological types of poliomyelitis virus. Because Col SK virus shares many of the properties of poliomyelitis virus, the former virus was also included in this study.

Materials and methods. A total of 57 red (*Ateles geoffroyi*) or black (*Ateles fusciceps robustus*) young spider monkeys, either captured in Panama or imported from Colombia, and weighing about 3-5 lb were used. To control the virulence of the test virus, a total of 29 rhesus monkeys were employed. Infection was carried out, as a rule, by intracerebral injection of 0.5 cc of a 1:10 virus suspension, made up from infected rhesus cords. The following strains of poliomyelitis virus were represented: Brunhilde, J.V.(7), Mahoney (Type I); Aycock, MEF_1 (Type II); Leon, Farabaugh (Type III).† Col SK virus was harvested from infected mouse brains. Following infection, the animals were observed for a period of 3-4 weeks. Monkeys developing paralysis were sacrificed and histological cord sections were examined for the presence of lesions. In some instances, infected spider cords were further passed to new spider or rhesus monkeys. Monkeys which survived without paralytic symptoms were reinfected with another type-strain of poliomyelitis virus.

Normal spider monkey serum, collected before infection, was tested for the presence of neutralizing antibodies against Brunhilde (Type I), Aycock (Type II), and Leon (Type III) virus. In these tests, one cc of pooled undiluted serum was mixed with one cc of a 1:10 viral cord suspension of each of the 3 strains and one cc of the mixtures, following incubation for one hour at 37°C, was injected intracerebrally into pairs of rhesus monkeys. Controls with normal rhesus monkey serum accompanied this test. A group of 40 individual specimens of normal spider monkey serum was also tested for neutralizing antibodies against Col SK virus. The test was carried out by mixing 0.5 cc of undiluted serum with 0.5 cc of decimal virus dilutions, ranging from 10^{-4} to 10^{-6}, and injecting the incubated serum-virus mixtures intraperitoneally in doses of 0.2 cc into groups of 4 mice for each level tested. A virulence control of the virus from 10^{-4} to 10^{-8} dilution, mixed with normal rhesus monkey serum, was added to each test.

Results. Table I shows that 3 Type I strains of poliomyelitis virus were equally

* Aided by a grant from the Sister Elizabeth Kenny Foundation.

† We are indebted to Dr. Jonas E. Salk, of University of Pittsburgh, for supplying us with the Mahoney, MEF_1 (monkey line) and Farabaugh strains.

TABLE I. Susceptibility of Ateles Monkeys and Rhesus Monkeys to Infection with Poliomyelitis and Col SK Virus.

Virus strain	Ateles monkeys						Rhesus monkeys					
	No.		Paralysis		No paralysis		No.		Paralysis		No paralysis	
Type I:												
Brunhilde	6	*4*	6	*3*	0	*1*	4	*2*	4	*2*	0	*0*
J.V.	4		4		0		2		2		0	
Mahoney	2		1		1		2		1		1	
	12	*4*	11	*3*	1	*1*	8	*2*	7	*2*	1	*0*
Total	16		14		2		10		9		1	
Type II:												
Aycock	8	*2*	0	*0*	8	*2*	3	*1*	3	*1*	0	*0*
MEF_1	3	*4*	0	*0*	3	*4*	2	*2*	2	*2*	0	*0*
	11	*6*	0	*0*	11	*6*	5	*3*	5	*3*	0	*0*
Total	17		0		17		8		8		0	
Type III:												
Leon	7	*4*	0	*0*	7	*4*	3	*2*	3	*2*	0	*0*
Farabaugh	3	*4*	0	*0*	3	*4*	2	*2*	2	*2*	0	*0*
	10	*8*	0	*0*	10	*8*	5	*4*	5	*4*	0	*0*
Total	18		0		18		9		9		0	
Col SK (para-polio)	4	*2*	4	*2*	0	*0*	2		0		2	
Total	6		6		0		2		0		2	

Figures in standard print refer to work in New York; figures in italics refer to work in Panama. Dose: 0.5 cc 10^{-1} i.c.

pathogenic for Ateles and rhesus monkeys, 14 of 16 of the former and 9 of 10 of the latter developing flaccid paralysis, with involvement of one or several limbs, within an incubation period of from one to 3 weeks. The spinal cords of the paralyzed spider monkeys showed typical poliomyelitic lesions which could not be distinguished from those found in rhesus monkeys. By contrast, none of 17 spider monkeys injected with 2 Type II strains and none of 18 spider monkeys injected with 2 Type III strains showed any signs of illness; all rhesus monkeys injected simultaneously with these strains, *i.e.*, 8 of 8 with Type II and 9 of 9 with Type III, developed paralysis. Six spider monkeys injected with Col SK virus all developed more or less extensive paralysis within 5-10 days following infection; 2 similarly injected rhesus monkeys remained free from symptoms. The cord lesions in the paralyzed spider monkeys were indistinguishable from those previously described in cynomolgus monkeys(8) and agreed in all histological details with the lesions induced in spider monkeys by infection with Type I poliomyelitis virus. The results obtained with the different strains of virus in the 2 laboratories closely paralleled each other.

The results of the reinfection experiments are brought together in Table II. In all, 20 spider monkeys which had resisted infection with either Type II or Type III poliomyelitis virus were reinfected, about one month later, with spider-pathogenic Type I poliomyelitis virus, the challenge strain being either Brunhilde or J.V. virus. The control animals for these tests are included in Table I. Previous infection with Type III virus (Leon, Farabaugh) afforded almost complete protection, and previous infection with Type II virus (Aycock, MEF_1) afforded partial protection against reinfection with the Type I Brunhilde strain (Table II). On the other hand, no protection was observed in either group against reinfection with the type I J.V. strain.

Neutralization tests showed that the normal spider monkey serum contained no antibodies against Types I, II or III poliomyelitis virus or Col SK virus which were demonstrable with the technics used in this work.

Discussion. The reported observations demonstrate a state of selective susceptibility in Ateles monkeys to infection with Type I poliomyelitis virus and with Col SK virus. To

TABLE II. Reinfection Experiments in Ateles Monkeys.

Previous asymptomatic infection			Reinfection with Type I poliomyelitis virus									
			Brunhilde						J.V.			
Strain	No.		No.		Paralysis		No paralysis		No.		Paralysis		No paralysis	
Type II:												
Aycock	3	*2*	2		1		1		1	*2*	1	*2*	0	*0*
MEF_1	2	*2*		*2*		*1*		*1*	2		2		0	
Type III:												
Leon	4	*4*	2	*4*	1	*0*	1	*4*	2		2		0	
Farabaugh	1	*2*		*2*		*0*		*2*	1		0		1	
	10	*10*	4	*8*	2	*1*	2	*7*	6	*2*	5	*2*	1	*0*
Total	20		12		3		9		8		7		1	

Figures in standard print refer to work in New York; figures in italics refer to work in Panama. Dose: 0.5 cc 10^{-1} i.c.

our knowledge, this is a unique phenomenon since all 3 serological type strains of poliomyelitis virus are highly virulent for other species of monkeys, including *M. rhesus* and *M. cynomolgus*. That more subtle differences may exist, however, between the 3 antigenic types (CNS material) with respect to their ability to infect cells is indicated by the observations of Youngner(9) who found that Type I strains, as a group, produce cytopathogenic changes more readily in monkey testis tissue culture roller tubes than do Type II or Type III strains. Inasmuch as a preliminary search for the presence of type-specific antibodies in the normal spider monkey serum gave negative results, the possibility of a previous immunizing exposure during the jungle life of these monkeys to Type II and Type III virus seems remote. The reasons for the refractory state are, therefore, entirely obscure. Equally unexplained is the fact that previous asymptomatic infection with Type II and Type III virus conveys a measure of non-specific cross protection against reinfection with Type I virus. A possibility exists that this phenomenon is caused by interference which has already been demonstrated in tissue culture medium between the 3 antigenic types of poliomyelitis virus(10). It is hoped that further investigation will throw more light on these problems.

Summary and conclusions. A group of 57 spider monkeys (*Ateles geoffreyi* or *Ateles fusciceps robustus*) from Central and South America were studied for their susceptibility to infection with poliomyelitis virus and Col SK virus. The animals were found to be highly susceptible to infection with 3 strains of Type I poliomyelitis and Col SK virus but were completely refractory to infection with 2 strains each of Type II and Type III poliomyelitis virus. Asymptomatic infection with Type II or Type III poliomyelitis virus conferred various degrees of protection against reinfection with one strain of Type I poliomyelitis virus.

It is a pleasure to express our appreciation of the skillful assistance of Mr. F. Vasi in our work.

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Growth of Human Embryonic Tissues in Cortisone-treated Laboratory Animals.*† (21178)

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Previous papers have reported the subcutaneous growth and serial transfer of human neoplasms and normal tissues in x-irradiated and/or cortisone-treated laboratory animals (1-5). Human embryonic tissues have been implanted also in cortisone-treated small animal hosts and successfully maintained, as will be noted herein. Although propagation of human embryonic tissues in heterologous hosts is not new since Greene(6) has reported growth of such material in the anterior chamber of the rabbit eye, the following preliminary observations are presented as offering a comparatively simple and easily reproducible procedure for growing human embryonic lung and other embryonic tissues in rats and hamsters.‡

Materials and methods. Six human embryos derived from therapeutic abortions and varying in age from 10-16 weeks furnished the material for this study. All tissues were received within 2 to 6 hours after operation of the maternal donor. Samples of lung and cartilage were obtained in 6 cases; skin in 4, intestine and trachea in 2, and liver in one. As soon as available, the tissues were minced with scalpels, suspended in a buffered Locke-Ringer's solution (ordinary isotonic saline was also satisfactory), and implanted subcutaneously in the flank of weanling rats x-irradiated 1-3 days previously with 150 r total body x-irradiation. The number of rats implanted varied from one to 6, depending on the amount of tissue. Immediately after implantation, the animals were injected subcutaneously in the nape of the neck with 3 mg of cortisone. Three similar doses of this drug were given on alternate days following: *i.e.*, if the implantation and first injection were on a Monday, they received subsequent injections on the next Wednesday, Friday and Monday.§ Two weeks after implantation, the rats were sacrificed and (in the case of the last 3 embryos received) the best growths removed and cut into pieces of approximately 25-40 mg each. These were implanted, according to the method of Lutz *et al.*(8), in the cheek pouches of anesthetized (nembutal) weanling, non-irradiated hamsters which received 3 mg of cortisone subcutaneously at the time of implantation and one mg about every 5th day following. The tissues were allowed either to remain in these hamsters or they were transferred to other hamster hosts similarly treated or to another group of x-irradiated and cortisone-treated rats. In some instances both the first and second generations were placed in rats and in one case embryonic lung was transferred for 7 generations (100 days) in rats alone. However, for reasons which will be given, the optimal procedure was found to be to use the rat as a "screening" host and the hamster thereafter. Details of all the methods and materials mentioned herein have been described previously(3,5).

The implanted tissues could be examined at any time by anesthetizing the hamster hosts, pulling out the pouches and viewing the contents thereof.

Results. All of the human embryonic tissues which were implanted survived and grew in the x-irradiated and cortisone-treated rats

* This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute of the U. S. Public Health Service, the American Cancer Society, and the Damon Runyon Memorial Fund for Cancer Research.

† The cortisone acetate (Cortone, Merck) used for these experiments has been supplied through the courtesy of Merck and Co., Rahway, N. J.

‡ A recent note has appeared by Handler, and Yerganian(7) on the growth of cartilage and brain from a 40 mm human embryo in cortisone-treated hamsters.

§ If radiation facilities are not available, non-irradiated rats can be used if the cortisone dosage, given in the manner described, is increased from 3 to 6 mg per injection.

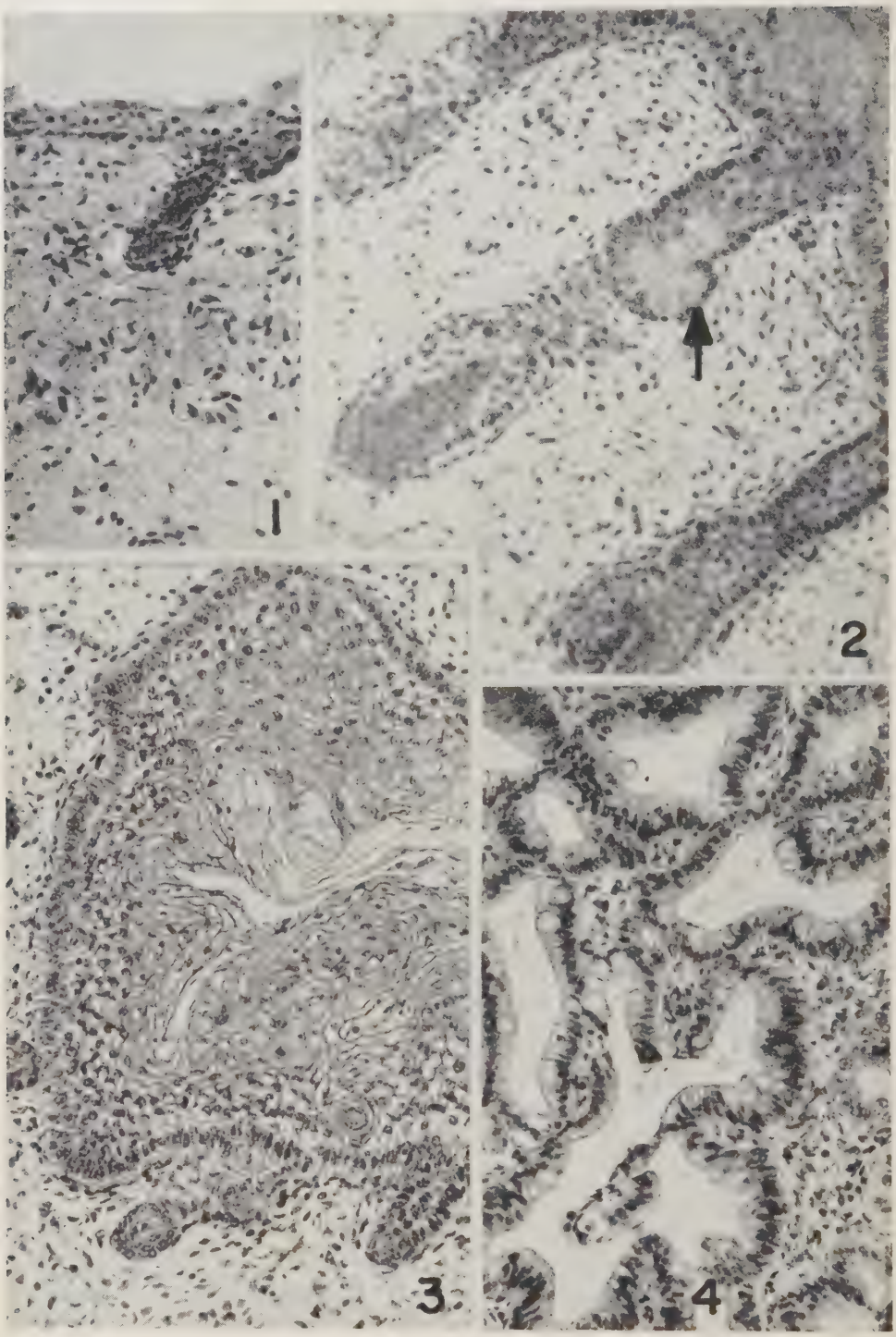


FIG. 1. Original skin obtained from a 16-week-old human embryo. A primitive hair follicle is present. ×160.

FIG. 2. Skin from embryo of Fig. 1 after 2 generations (27 days) in the subcutaneous tissues of x-irradiated rats treated with cortisone (generation 1 = 13 days; generation 2 = 14 days). Hair

follicles are well developed and arrow points to sebaceous gland. ×320.

FIG. 3. Cyst of human embryonic skin in the same animal as Fig. 2. Note multiple layers and differentiation. ×160.

FIG. 4. Embryonic intestine of a 16-wk human embryo after 2 generations (47 days) in cortisone treated laboratory animals (generation 1 = 14 days in x-irradiated rat; generation 2 = 33 days in hamster's pouch). Goblet cells are prominent. ×160.

with the exception of the one sample of liver. Varying degrees of differentiation occurred in the first and subsequent transfer generations. Skin which was 3-4 layers thick with a few primitive hair follicles in the original embryo (Fig. 1) developed, after 27 days in rats, into well differentiated integument (Fig. 2 and 3) with mature hair follicles and associated sebaceous glands. Intestine, in the 2 samples observed for 30-47 days, did not change much in character (Fig. 4) although there was a tendency for this material to produce cysts which were lined by intestinal cells piled upon one another and sloughing into the central areas. Such formations were obviously a result of the abnormal environmental conditions. Areas of healthy proliferating smooth muscle cells were often seen in these implants.

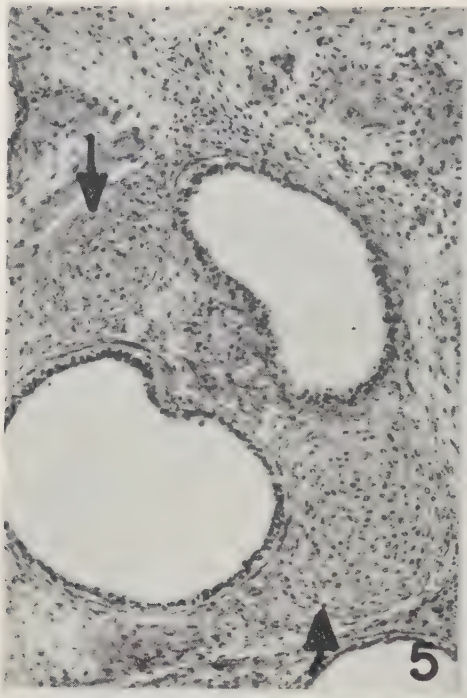
Cartilage grew very well in all the animals and gradually became calcified (Fig. 6). Of note was the finding that primitive cartilage appeared in association with embryonic trachea or lung bronchioles (Fig. 5,10) long after the initial implantation of these tissues. Apparently it was formed from the embryonic mesenchyme or connective tissue in which mitotic figures were common. Typical bone formation by osteoblasts was observed (Fig. 7).

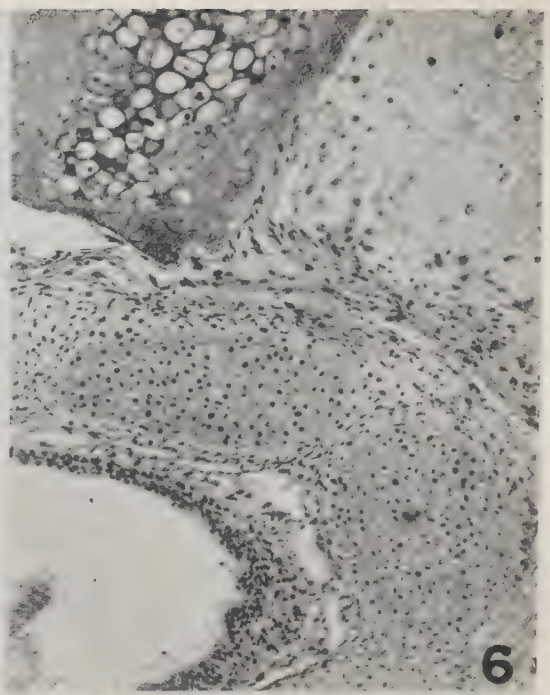
Of the tissues implanted, embryonic lung apparently underwent the most immediate comparative maturation. The immature typically fetal lung composed of small lobules imbedded in a mass of spongy connective tissue (Fig. 8) changed to a postnatal type of lung tissue after one generation in the animal hosts (Fig. 9-11). As far as could be determined, this picture was produced by a dilatation of all the lobules and bronchioles with fluid from the host animal. After this initial alteration, further changes were less startling although the lung tissues grew slowly but progressively whether maintained in rats for 7 generations (100 days) or, in another case, implanted in hamsters for 43 days after 2 generations in rats (27 days).

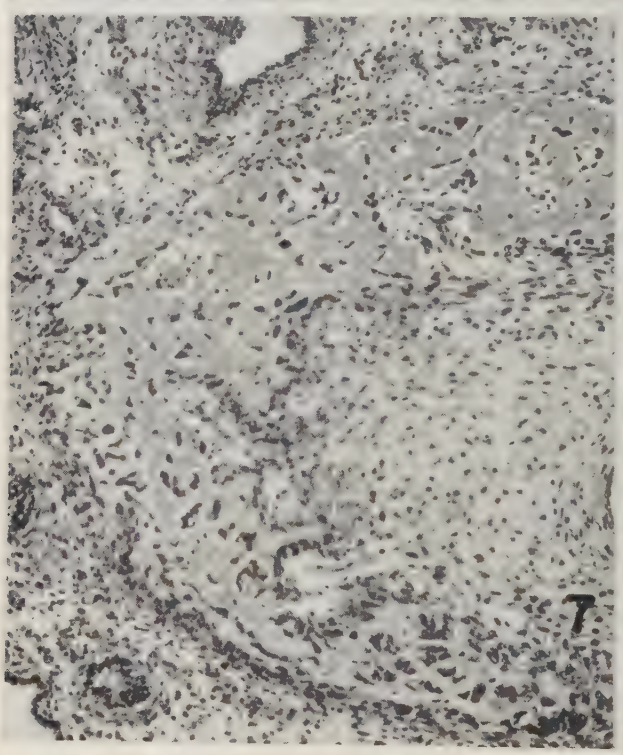
Complete descriptions of the different types of host-transfer combinations which were employed after the initial generation in rats, would entail detailed charts for each embryonic tissue specimen implanted: *i.e.,* transfer to the second generation was often in both rats and hamsters and implanted tissues from these, in turn, might supply either rats or hamsters or both in the third. Such manipulation led to the conclusion that the simplest and most effective method for growing embryonic tissues is to make all initial implantations in rats, a procedure which takes less time and skill than implantation into the hamster pouch. From the implanted embryonic material harvested 14 days later, a selection is then made of obviously choice areas. These can be cut up for placement in the hamster pouch wherein they may stay over long periods of time—thus furnishing excellent material for experimental use which can be viewed at any time. Further, the embryonic tissues do not increase in mass in the manner of the rapidly growing transplantable tumors(4,5) so that it is desirable to have a site such as the hamster pouch in which they can remain and develop. Constant transfer every 14 days from rat to rat is traumatic to the tissues and causes a loss which, for slow growing tissues, is of import.

Since this study is as yet in its preliminary stages, it is not known just how long the embryonic transplants can be maintained. Material from the first 3 embryos obtained was discontinued for microscopic examination after 47-100 days in laboratory animals. Lung, cartilage, skin, and intestine from the other 3 embryos are still being carried by cortisone-treated hamsters and, in some instances, x-irradiated rats. The oldest of these is now 8 weeks and is in good condition. It would seem that a long term experimental procedure, employing this material, is feasible.

It should be noted that samples of all the embryonic material received were implanted







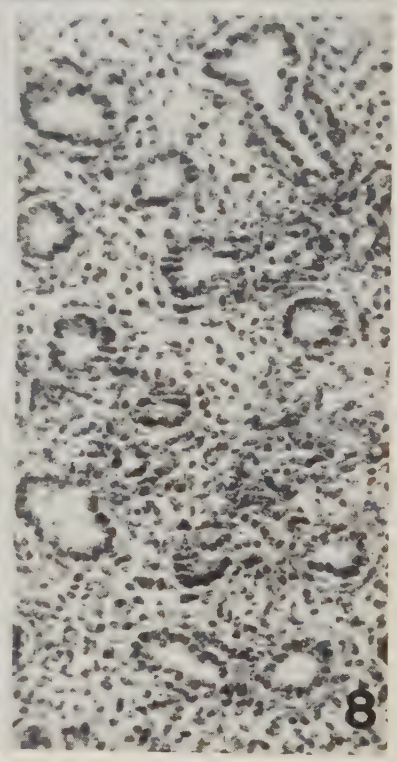


FIG. 5-7. Implants of minced human embryonic chest cage (trachea plus small amount of lung, rib and humerus from a 12-wk embryo) after 2 generations (28 days) in x-irradiated rats. Fig. 5 shows primitive cartilage (arrows) associated with embryonic bronchioles. In Fig. 6 trachea with its surrounding young cartilage is at bottom of picture and pieces of cartilage in various stages (probably derived from humerus) are above. Fig. 7 shows bone formation by osteoblasts. All $\times 90$.

FIG. 8. Original embryonic lung from 16-wk human embryo. Note small tubules lined by columnar epithelium and imbedded in a connective tissue matrix. $\times$ 160.

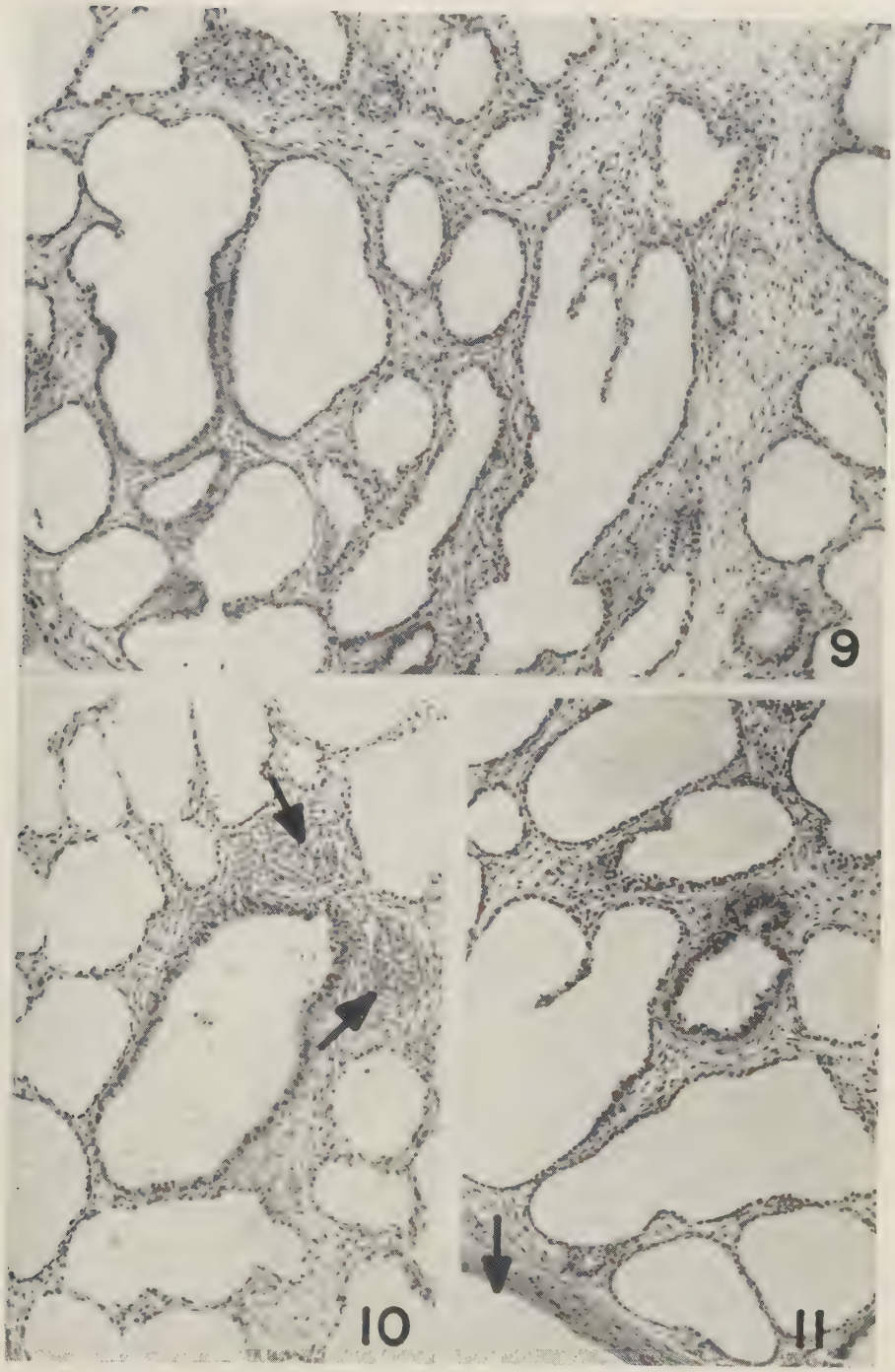


FIG. 9-11. Same lung as Fig. 8 after 47 days in cortisone-treated animals (G1 = 14 days in an x-irradiated rat; G2 = 33 days in hamsters). Each of these tissues was removed from a different hamster. Fig. 9 shows post natal appearance of dilated lobules. Fig. 10 again demonstrates primitive cartilage associated with a bronchiole and apparently derived from surrounding mesenchyme. Fig. 11 illustrates how little host reaction toward human tissue occurs (arrow points to edge of implant in hamster pouch). All ×90.

in roller tube tissue culture‖ at the same time they initially were implanted in rats. After the first generation of growth in these animals (and often after 2 or 3 generations of transfer), some of the harvested embryonic material was again placed in tissue culture. Without exception, much better *in vitro* growth was obtained from the rat grown tissue than the original material. The possible explanation for this finding will be discussed elsewhere(5).

Summary. Human embryonic tissues particularly lung, skin, cartilage, and intestine have been propagated in x-irradiated rats or nonirradiated hamsters both treated with cortisone. The method of growing these tissues is simple and so uniformly reproducible that it offers a good source of human embryonic material for experimental use and study.

‖ All tissue cultures were done in the laboratory of Dr. Alice Moore of The Sloan-Kettering Institute.

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Preparation, Characterization, and Antigenicity of a Saline Soluble Fraction From Shigella Types. (21179)

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Several investigators have extracted and characterized the somatic antigen of *Shigella sonnei* and the *Sh. paradysenteriae* (Flexner) group(1,2). The complete antigen appears to be a complex of polysaccharide, phospholipid, and protein. The polysaccharide of the antigenic complex determines specificity and it can elicit antibodies in man and the mouse, but not in the rabbit. The protein fraction appears to be the most toxic component of the complex. Numerous attempts have been made to reduce the toxicity of the complete antigen(3) but decreased antigenicity usually accompanies reduced toxicity.

Vaccines made of whole cells have been used in prophylactic immunization against bacillary dysentery. Most preparatory methods(4,5), and most extraction methods for somatic antigens, include the washing of cells with saline solution, an act that may cause a significant loss of antigen from the cells. The successful extraction of Vi antigen from *Escherichia coli* with isotonic saline has recently been reported (6,7). A similar method was used here for the extraction of shigellae. This report concerns the preparation, preliminary analyses, and some immunogenic properties of saline soluble antigens from 3 *Shigella* types.

Materials and methods. The cultures used in this study were *Sh. sonnei*, strain (Ch); *Sh. paradysenteriae, Flexner III*, strain (Be); and *Sh. paradysenteriae, Flexner II*, strains (Km) and (PR).* The organisms were grown in a chemically defined medium (Table I). The (Km) strain of Flexner II required 17 g of dibasic phosphate and 5 g of glucose per liter of this medium. Cultures were incubated with shaking for 24 hours at 37°C in liter quantities. The cultures were centrifuged and the packed cells resuspended in cold acetone and dried on filter paper. One liter of the chemically defined medium yielded approximately 2 g of dry cells.

* Obtained through the courtesy of Dr. M. L. Cooper, The Children's Hospital Research Foundation, Cincinnati, O.

TABLE I. A Chemically Defined Medium for Cultivation of Shigella Species.

L-glutamic acid	3.4 g
L-aspartic acid	2.8
L-lysine	.5
DL-valine	.4
DL-serine	.4
L-leucine	.25
L-histidine	.2
DL-threonine	.16
DL-methionine	.13
DL-isoleucine	.1
L-arginine	.05
L-cysteine	.05
L-proline	.05
L-tyrosine	.03
DL-phenylalanine	.015
Glycine	1.0
$K_2HPO_4 \cdot 3\ H_2O$	12.25
NaCl	8.0
Nicotinamide	5.0 mg
$MgSO_4 \cdot 7\ H_2O$	50.0 "
Glucose	10.0 g
Distilled water	1000.0 ml

(Adjusted to pH 7.3-7.4 with 10% NaOH.)
Glucose added after sterilization.

Preparation of extract. One gram of acetone dried cells was added to 100 ml of sterile 0.9% saline previously adjusted to pH 4.0 with N/1 HCl. This cell suspension was shaken at 37°C for 30 minutes and then centrifuged at 3000 rpm for one hour and the supernatant fluid removed. If the fluid was not clear at this point it was recentrifuged at 10,000 rpm for 30 minutes. The clear supernatant was placed in cellulose casing (Visking) and dialyzed overnight in running tap water. Then the extract was filtered through a Seitz filter and stored in sterile vaccine vials at 4°C. Extracts were thus prepared from cells of *Sh. sonnei* (Ch), Flexner III (Be), and Flexner II (Km). A polyvalent extract was prepared by mixing 10 ml of each extract and bringing the volume to 100 ml with saline.

Mouse protective potency tests. The antigenicity of the extracts was determined by an active immunization mouse protective potency test based upon a constant immunizing dose and a graded challenge. Albino Swiss-Webster mice† of both sexes weighing between 15 to 18 g were used in all tests. Mice were given an immunizing dose of 0.1 ml of the respective extract or a dilution thereof by the intraperitoneal route. Seven days later the mice were challenged with graded doses of the homologous or heterologous strain of *Shigella*. The challenge culture was removed from a semisolid brain heart infusion stock and given at least five 12-hour subcultivations in trypticase soy broth (BBL), supplemented with 5 μg/ml nicotinamide and 7 mg/ml KH_2PO_4. The last 12-hour subculture for challenge was diluted to a standard turbidity reading equal to approximately 2 billion/ml viable cells. This suspension was diluted 1:5 and serial 10-fold dilutions made thereafter. A portion of each dilution was mixed with an equal quantity of sterile 6% hog gastric mucin.‡ Plate counts were made on 2 appropriate dilutions before the addition of mucin to determine the number of organisms in the LD_{50} dose. Each mouse received a challenge dose of 1.0 ml intraperitoneally. The mice were observed for 5 days. The 50% end points were computed according to the Reed and Muench method(8). The protective index was obtained by subtracting the reciprocal of the LD_{50} dilution of culture for immunized mice from the reciprocal of the LD_{50} dilution of culture for nonvaccinated mice. The index gives the logarithm of the number of LD_{50} doses that the mice resisted.

Toxicity tests. Early mortality in mice was noted at the time of immunization. Normal temperature and weight curves on guinea pigs were kept for 3 successive days after which graded amounts of the extracts were given intraperitoneally to each of 2 animals weighing between 350 to 500 g. Temperatures were recorded every 4 hours for the first 24 hours and then daily thereafter for one week. The weights were recorded daily for one week.

Chemical characterization. Preliminary chemical analyses of extracts included determination of nitrogen by the micro-Kjeldahl method(9), carbohydrates by either the Molisch or Dische method(9), and chromatographic tests for proteins, amino acids, simple sugars, and polysaccharides(10). n-Butanol-acetic acid-water (4:1:5, v/v) and buffered phenol solvents followed by n-butanol-ninhydrin spray was used to determine proteins and amino acids. n-Butanol-acetic acid-

† Obtained from Hamilton Laboratory Animals, Inc., Cincinnati, O.

‡ Type 1701-W obtained from Wilson Laboratories, Chicago, Ill.

TABLE II. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella paradysenteriae* (Flexner II) (Km).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$: 1	2	3	4	5	6	7	8	9	10	LD_{50} $1 \times 10^{-}$	Protective index
Flexner II (Km)													
Undiluted	Flexner II (Km)	5/7*	2/7	1/6	0/7	0/6						1.6	6.1
1: 10	"	4/7	1/7	1/6	0/7	1/7						1.4	6.3
1: 50	"	6/6	5/6	0/6	2/6	0/6						2.6	5.1
1:100	"	4/6	6/6	3/6	2/6	1/6						3.1	4.6
	" control					5/5	4/5	4/5	2/5	0/5	1/5	7.7†	—
Undiluted	Flexner III (Be)				5/5	5/5	3/5	3/5	3/5			7.4	1.7
	" control					4/5	4/5	5/5	5/5	4/5	0/5	9.1†	—
"	Sonnei (Ch)					6/6	5/6	6/6	4/6	6/6		>9.0	<.4
	" control					4/5	5/5	5/5	5/5	4/5	1/5	9.4†	—
"	Flexner II (PR)		5/6	3/6	1/6	1/6	1/6					3.3	6.0
	" control					5/5	5/5	3/5	4/5	4/5	2/5	9.3†	

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner II (Km), 9; Flexner III, 1; Sonnei, 1; Flexner II (PR), 1.

water (4:1:5) followed by alkaline permanganate or m-phenylenediamine spray was used for carbohydrate qualitative tests.

Results. The results of mouse protective potency tests on animals immunized with the 3 saline fractions are presented in Tables II to IV. The Flexner II extract induced protection in excess of one million LD_{50} doses against the homologous organism and against one million LD_{50} doses of the (PR) strain of Flexner II (Table II). There was little or no immunity against heterologous challenge. Essentially similar results were obtained with the Flexner III and Sonnei extracts. Following vaccination with the Flexner III extract (Table III) mice resisted between 7 and 8 logs of the homologous strain but only 2.6 logs and 0.7 log of the heterologous Flexner II and Sonnei strains, respectively. Following vaccination with the Sonnei extract (Table IV) mice resisted considerable amounts of the homologous strain but only 1.6 logs of the heterologous Flexner II and 3.9 logs of the heterologous Flexner III. The results also indicate that the extracts may be diluted as much as 100-fold with little loss in homologous antigenicity.

In Table V are shown the results after vaccination with the polyvalent extract. Mice developed good immunity against all 3 types of *Shigella* with protective indices of 6 logs or more against each organism.

Toxicity studies in mice with the undiluted extracts revealed the following mortality:

TABLE III. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella paradysenteriae* (Flexner III) (Be).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$: 1	2	3	4	5	6	7	8	9	10	LD_{50} $1 \times 10^{-}$	Protective index
Flexner III (Be)													
Undiluted	Flexner III (Be)	2/5*	1/5	0/5	0/5	0/6						1.0	7.8
1: 10	"	0/6	1/6	1/5	0/5	1/5						<.7	>8.1
1: 50	"	2/5	1/6	1/6	0/6	2/6						1.5	7.3
1:100	"	1/5	1/5	0/6	1/5	0/5						<.9	>7.9
	" control					5/5	5/5	5/5	5/5	2/5	0/5	8.8†	—
Undiluted	Flexner II (Km)		4/5	5/5	4/5	4/5	4/5	4/4				7.0	2.6
	" control					5/5	5/5	5/5	5/5	5/5	1/5	9.6†	—
"	Sonnei (Ch)					4/5	4/5	5/5	4/5	4/5	1/5	9.1	.7
	" control					5/5	5/5	5/5	5/5	5/5	2/5	9.8†	—

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner III control, 1; Flexner II control, 1; Sonnei control, 1.

TABLE IV. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella sonnei* (Ch).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$: 1	2	3	4	5	6	7	8	9	10	LD_{50} $1 \times 10^{-}$	Protective index
Sonnei (Ch)													
Undiluted	Sonnei (Ch)	3/7*	3/7	0/7	1/5	1/6						1.6	7.9
1: 10	"	7/7	1/7	1/7	1/7	0/7						1.7	7.8
1: 50	"	6/6	1/6	0/6	2/6	1/6						1.9	7.6
1:100	"	4/6	5/6	3/6	0/6	1/6						2.7	6.8
	" control					5/5	5/5	4/5	5/5	4/5	2/5	9.5†	—
Undiluted	Flexner II (Km)					3/6	0/6	1/6	1/5	1/5	0/5	5.4	1.6
	" control					5/5	2/5	3/5	1/5	1/5	0/5	7.0†	—
"	Flexner III (Be)			4/5	1/5	3/5	3/5	2/5				5.1	3.9
	" control					5/5	5/5	5/5	4/5	3/5	0/5	9.0†	—

* Numerator denotes No. of deaths; denominator, total mice tested.

† No. of organisms in LD_{50} dose: Sonnei control, 1; Flexner II (Km) control, 14; Flexner III control, 1.

Flexner II, 4.6%; Flexner III, 19%; and Sonnei, 5.2%. Diluting the extracts 1:10 removed the toxic components of the Flexner II and Sonnei extract but still gave a 10% mortality with the Flexner III extract. No deaths were observed when any one of the extracts was diluted 1:50 or above. The number of mice tested ranged from 30 to 100 for each dilution. A close correlation was observed when the extracts were given to guinea pigs. Doses of 1.0 ml or 5.0 ml of the Flexner II and Sonnei extract caused only a slight febrile reaction and some transient weight loss while 5.0 ml of Flexner III extract was lethal to 2 guinea pigs in 8 hours. The polyvalent vaccine composed of 1:10 dilutions of each of the 3 extracts was lethal to only 9/421 (2.1%) of mice immunized. No untoward reactions were observed in guinea pigs receiving 5.0 ml of the polyvalent extract.

The extracts were found to contain only small amounts of nitrogen. Dialysis reduced the nitrogen content of the extracts approximately one-half. Final nitrogen values of the extracts (mg % N) were: Flexner II, 19.2; Flexner III, 11.2; and Sonnei, 11.6. The polyvalent extract contained 3.02 mg N/100 ml. There was no chromatographic evidence of protein or free amino acids. Concentration of 5.0 ml amounts to 0.2 ml did not give chromatographic evidence of free amino acids. The extracts gave positive Molisch tests. The Dische quantitative carbohydrate test has been performed on the Flexner II extract. Small amounts of carbohydrate (16 mg/100 ml extract) were found. Chromatographic analysis failed to reveal any free simple sugars.

Discussion. The results presented demonstrate a significant immune response in mice following injection of a saline soluble fraction from *Shigella* cells. The heterologous protection afforded by the 3 saline extracts is low but homologous protection exceeds a million LD_{50} doses in all instances. The polyvalent extract is reduced in toxicity but still gives significant cross protection against the 3 strains of *Shigella*. With the use of female mice, where rapid weight increases do not occur over a week's time, protective indices of 100 million LD_{50} doses have been obtained. It is felt that the toxicity of the polyvalent extract resides in the Flexner III extract and that mouse mortality could be further reduced by increasing the dilution of Flexner III extract.

The extraction procedure is rapid and subtle and does not seem to remove a great deal of the cell wall material or diffusible cell constituents. The extract appears to contain a nondialyzable polysaccharide consisting of possibly an amino sugar. Slein and Schnell (11) in recent studies on a polysaccharide isolated from Flexner III have identified an acetylated amino sugar (D-glucosamine), rhamnose, glucose, and phosphorus. Further chemical studies are in progress to identify and purify the active fraction. In addition,

TABLE V. Summary of Immunity Tests with Polyvalent Saline Extract of Flexner II, III, and Sonnei. (Final dilution of 1:10 for each extract.)

Challenge culture	Challenge dilution $1 \times 10^{-}$										LD_{50} $1 \times 10^{-}$	Protective index
	1	2	3	4	5	6	7	8	9	10		
Flexner II (Km)	6/6*	4/6	1/6	1/6	0/6	1/6					2.6	6.0
" control					5/5	5/5	5/5	5/5	0/5	1/5	8.6†	—
" (PR)	4/5	0/5	3/5	1/5	1/5						1.9	6.8
" control					5/5	2/5	5/5	5/5	2/5	2/5	8.7†	—
Flexner III (Be)	4/5	0/5	1/5	0/5	1/5	1/5					1.7	6.8
" control					5/5	5/5	4/5	4/5	1/5	1/5	8.5†	—
Sonnei (Ch)	3/5	2/5	3/5	2/5	0/5	0/5					2.5	6.9
" control					5/5	5/5	5/5	4/5	5/5	0/5	9.4†	—

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner II (Km) control, 5; Flexner II (PR) control, 5; Flexner III control, 5; Sonnei control, 1.

further work is in progress to determine the antigenicity of the extracts in rabbits and to investigate the activity of the polyvalent extract when given by other immunizing routes to the mouse.

Summary. A procedure for the extraction of a saline soluble fraction from *Shigella* species is described. Each of the 3 extracts protects mice against massive challenge with the homologous strain of *Shigella*. Heterologous protection is of low order or insignificant. A 1:10 dilution of the extracts reduces the toxicity while retaining the essential protective properties. A polyvalent extract has been prepared containing a 1:10 dilution of each of the 3 extracts. Preliminary chemical characterization indicates a nondialyzable carbohydrate-containing compound with small amounts of nitrogen present.

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Metabolic Effects of the Pancreatic Hyperglycemic Factor. (21180)

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Following the demonstration by Thorogood and Zimmerman(1) that pancreatectomy decreased the insulin requirement but increased the ketonuria of alloxan-diabetic dogs, attention has turned to the metabolic significance of glucagon, the pancreatic hyperglycemic glycogenolytic factor (HGF). There is general agreement as to its effect on blood sugar and liver glycogen, but recent work on its role in fat metabolism has given conflicting results. Indirect evidence such as that derived from the experiment of Thorogood and Zimmerman indicated that HGF was an antiketogenic substance. Stewart and Roitman(2) found that pancreatic extracts similar to those containing HGF did inhibit the increase in ketogenesis usually produced in liver slices by the addition of anterior pituitary extract. Haugaard found that HGF increased the ketogenesis by liver slices from glucose, but interpreted this as an effect secondary to an inhibition of lipogenesis from the glucose(3). *In vivo* experiments have been similarly equivocal. Foa(4) reported that insulin-free pancreatic extracts decreased the ketonemia of depancreatized dogs, while purified HGF produced an increase; it was concluded that the pancreatic extract contained an antiketogenic material other than insulin or HGF. Unfortunately this report does not make the experimental data available so that their quantitative significance cannot be judged. Pincus(5) found no effect of HGF on ketonemia in depancreatized dogs, while Zimmerman and Donovan (6) observed a slight increase followed by a more marked decrease when inactivated commercial insulin was given to such animals.

HGF has been reported to increase nitrogen excretion in acute experiments on rabbits (7); unfortunately the data as presented are not subject to statistical analysis.

The following work was undertaken in an attempt to assess the effect of HGF on fasting metabolism, and more specifically on fasting ketosis.

Method. Male hooded rats weighing 210-230 g were fasted for 24 hours. Blood was taken from the jugular vein, and the animals were then given an intraperitoneal injection of 33 γ of HGF in dilute NaOH solution (pH 9.5). Two more such injections were given at intervals of one hour. Four hours after the initial injection a second sample of blood was obtained from the jugular vein. All blood specimens were analyzed for total ketone content by the method of Lester and Greenberg(8).

A second group of rats received HGF injections of 50 γ each at 6, 10, 14 and 22 hours after the onset of fasting. Blood was taken after 24 hours of fasting.

A third group of animals was treated in the same manner as group two, but the total dose of HGF was 600 γ/day. Blood was then taken for sugar determinations(9) and samples of liver for glycogen content(10).

Control animals for these 3 groups received injections of the NaOH solution used as solvent for the HGF.

A fourth group of animals was placed in metabolic cages, and fasted for 24 hours, receiving only injections of the NaOH solution. The urine was analyzed for total nitrogen. A week later, when the rats had regained their initial weight and shown some increase as well, they were again fasted, this time getting injections of 600 γ of HGF in divided doses at 4, 10, and 15 hours after the start of the fast. The urine was again analyzed for total nitro-

TABLE I. Acute Effects of HGF on Ketonemia and Glycemia.

	4-hr change in ketonemia, mg%	4-hr change in glycemia, mg%
Control	(8) $+1.0 \pm .6$	(8) $+18.0 \pm 4.3$
HGF	(8) $-1.5 \pm .25$	(9) $+18.6 \pm 3.7$
	$p < .01$	$p > .05$

Figures in parentheses indicate the No. of animals used: Values shown are Mean $\pm$ S.E.M.

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TABLE II. Effects of HGF on Fasting Metabolism.

	Ketonemia,* mg%	Glycemia, mg%	Urine N, mg	Liver glycogen, %	Mouse liver fat, %
Control	(6) 7.3 ± .21	(21) 108 ± 2.2	(7) 212 ± 16.8	(8) .05 ± .02	(4) 7.2 ± .28
HGF	(8) 3.9 ± .45	(7) 102 ± 4.0	(7) 295 ± 9.6	(8) .12 ± .03	(4) 6.3 ± .17
	$p < .01$	$p > .05$	$p < .02$	$p > .05$	$p < .05$

* Ketonemia in fed state is 2.5 ± .2 mg%.
Figures in parentheses indicate the No. of animals used.

gen. The use of each animal as its own control overcomes the great individual variations in nitrogen excretion known to occur in these animals.

The effect of HGF on liver fat was measured in mice according to the assay procedure of Campbell(11). The dose used was 125 γ given subcutaneously.

The HGF preparation used was Lot No. 208-158B-197 of the Lilly Research Laboratories, supplied through the courtesy of Dr. W. R. Kirtley. This material contains only traces of insulin or none at all; 0.2 γ per kilo given intravenously to an anesthetized cat produces an optimal rise in blood sugar(12).

Results and discussion. The results of these experiments are shown in Tables I and II.

It is seen that HGF caused a reduction in ketonemia over a 4-hour period, and greatly inhibited the ketosis developed by a day of fasting. The acute effect on ketonemia is in agreement with results obtained by Zimmerman and Donovan(6) and dismissed by these workers as an effect of residual "hypoglycemic activity"; this interpretation appears unjustified both from their own data, since the fall in ketonemia occurred often with no accompanying fall in glycemia, and from the present results obtained with a highly purified material. At the same time there was an increase in nitrogen excretion indicating an increase in protein catabolism. These changes together with the slight decrease in liver fat indicate that HGF caused an alteration in energy metabolism so that less fat and more protein were used, in comparison with the control rats. This interpretation is supported by a preliminary report of Waters(13) that insulin contaminated by HGF produced an increase in gluconeogenesis by liver slices.

It is interesting that the liver glycogen and blood sugar were not increased above the control levels. This is in contrast to the changes brought about by cortisone, which in a dose of 10 mg given in divided doses during a day of fasting, caused a less marked inhibition of ketonemia, but a definite rise in liver glycogen. These effects are shown by the results obtained in groups of 8 animals (Table III). The difference in the effects on liver glycogen may be a quantitative one, due to a greater protein catabolism by cortisone with comparably increased gluconeogenesis; this is improbable in view of the weaker antiketogenic effect. Thus it is likely that the changes brought about by HGF are not mediated by the adrenal hormones and those brought about by cortisone are not mediated by HGF. In any case HGF appears as still another hormonal factor to be considered in problems of protein (and fat) metabolism.

These results do not prove that the action of HGF is responsible for the failure of alloxanized animals to develop severe ketosis, but they are compatible with this concept.

An incidental implication of the results described above is that growth hormone does not produce its metabolic effects by acting through HGF, as has been suggested(14,15); growth hormone given to fasted animals causes a decrease in nitrogen excretion while HGF caused an increase. However it is conceivable that growth hormone exerts 2 opposing influences on protein catabolism, one via HGF,

TABLE III. Effect of Cortisone on Fasting Metabolism.

	Liver glycogen, %	Ketonemia, mg%
Cortisone	1.46 ± .29	5.0 ± .86
Control	.05 ± .02	6.7 ± .55

and that a preponderance of the latter effect in man accounts for the failure to obtain nitrogen retention with growth hormone in this species. This is entirely speculative since there is no direct evidence that growth hormone does stimulate the production of HGF.

Summary. Purified pancreatic hyperglycemic factor has been given to normal rats during a period of fasting or at the end of such a period. In the latter case HGF caused a fall in the level of ketonemia which had developed as a result of the fast, but did not affect the blood sugar. When given during the fast, HGF greatly inhibited the development of ketonemia, increased the nitrogen excretion, and inhibited to some degree the rise in liver fat; it did not affect the liver glycogen or the blood sugar. These effects differ from those of adrenal hormones and of growth hormone. It appears that HGF is capable of playing an active part in fasting metabolism.

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Influence of Smoking on Urinary Pepsinogen Excretion.* (21181)

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Whether smoking stimulates gastric secretion has not been definitely ascertained. The after-dinner smoke is believed to aid digestion and earlier workers reported that smoking stimulated gastric secretion and acidity. However, Schnedorf and Ivy(1) found that smoking produced no immediate effect on acid production but smokers had a higher gastric acidity (not statistically significant) than non-smokers. They reported that smoking had no effect on gastric evacuation but Dickson and Wilson(2) noticed a slight delay.

We have studied the effects of smoking on urinary pepsinogen excretion.

Method. To determine the excretion rate of urinary pepsinogen, the method of West, Ellis, and Scott(3) employing a casein substrate(4) was used. Twenty white male office and professional hospital workers between 18 and 45 years of age, who smoke 20 or more cigarettes per day, were examined on 5 not necessarily successive days. The results were compared with those of a control group of 20 non-smokers.

Results. The results are shown in Fig. 1. Aside from the diurnal variations(4) we found considerable day to day variation in healthy control individuals. These variations were not unexpected. They are usually within a limited range (though in rare instances they may exceed 100% of the mean value). These variations must be ascribed to the day to day

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FIG. 1. Urinary pepsinogen excretion in units per hr. Each vertical column represents 5 test results on one individual.

variety of life situations. Smoking was suspected of increasing gastric secretion, either directly or indirectly.

The units of urinary pepsinogen excreted per hour in the group of smokers averaged 43 compared to the non-smokers with an average value of 30. This means that the average urinary pepsinogen excretion of smokers was 43% higher than that of non-smokers. Testing the significance of the differences between the means of the 2 samples by the Fisher "t" test(5) yields a "t" of 2.34, which is significant at $P = 0.03$. Since the direction of the difference was hypothesized, the use of a one-tailed test yields a highly significant value of $\frac{P}{2} = 0.015$.

In this study no special attention was paid to the question of whether or not the smokers did smoke immediately prior to or during the collection period. The test persons were given no instructions and it is assumed that most of them did smoke during the time in question. A group of 5 smokers submitted each to 8 further tests. Alternately, they did not smoke on the test day until after the final urinary specimen was collected, or they smoked at least 3 cigarettes during the collection period. Uropepsin excretion during smoking averaged 47 units per hour, as compared to 43 during abstinence.

Comment. The results indicate that the immediate effect of smoking on the urinary pepsinogen excretion rate, though statistically significant, is not of appreciable magnitude. This agrees with the findings of Schnedorf and Ivy, who found no immediate effect on acid production. No quantitative pepsin determinations in relation to smoking have to our knowledge been previously done. No influence of smoking on duodenal proteolytic enzyme production has been found(6). The mechanism for the prolonged effect of smoking on pepsin secretion is not clear.

Summary. The average excretion of urinary pepsinogen of habitual smokers was found to be 43% higher than that of non-smokers.

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Correlation of Plasma Level with Spinal Cord Depressant Action of Mephenesin and its Carbamic Acid Ester.*† (21182)

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The action of mephenesin on multineuron reflexes has been studied intensively by a large number of investigators. Clinical applications have been hampered by the short duration of action of this drug and the consequent need to administer relatively large doses at frequent intervals. Several attempts have been made to increase the duration of action by esterification of the primary alcohol group of mephenesin which has been shown to undergo rapid oxidation *in vivo* to the inactive lactic acid(1). One of the compounds which was synthesized for this reason is the carbamic acid ester of mephenesin (hereafter referred to as the carbamate).

The carbamate(2-4) has been shown to remain in the circulation for a significantly longer period after oral or intravenous administration. The distribution of both compounds in dogs has also been studied(4,5) and their tissue plasma ratios at high blood levels has been found to be effectively the same. Some of the pharmacologic actions of the carbamate have been described previously(6). The great similarity of action of the 2 compounds by a number of criteria was pointed out. The carbamate was shown to be slightly more potent on a molar basis by some of these criteria. There seemed to remain a possibility that the increased duration of action could be a function of potency alone. The present work is an attempt to resolve this possibility.

Methods. Analytical. Plasma levels of mephenesin and its carbamic acid ester were determined by a modification of a method of Titus, Ulick, and Richardson(7). The modification was made in order to determine the low plasma levels dealt with in this work. 4-5 ml of blood collected from the carotid artery through a polyethylene catheter were oxalated immediately in 15 ml centrifuge tubes and, if necessary, stored in the refrigerator. One ml samples of plasma were transferred to glass-stoppered centrifuge tubes of 15 ml capacity. 2.5 ml of 0.4 N NaOH were added to each sample, followed by 6.6 ml of cold Reagent (Merck) Ethyl Ether. The tubes were stoppered immediately and vigorously shaken for 8 minutes in a shaking apparatus which allowed them to be held in the horizontal position. Five ml of the ether layer were transferred to another centrifuge tube using a 10 ml syringe. The ether was evaporated to dryness in a water bath held at 42°C. Three ml of Phosphoric Acid (Baker's Analyzed) and 0.4 ml of the same diazotized sulfanilic acid reagent used in the original method(7) were added to each tube and mixed by inversion. The color reaction proceeded in a boiling water bath for 15 minutes, at the end of which the tubes were cooled in ice water. The color was read as % transmission on a Lumetron colorimeter using an M 515 filter. Each set of samples was analyzed against a blank from blood taken before the beginning of the infusion and at least 3 concentrations of a standard solution of the compound were run to establish a standard curve. Each plasma sample was analyzed in duplicate. We consider this method to be accurate to $\pm$3-5 μg/ml plasma with sensitivity from 10-70 μg of mephenesin and from 15-80 μg of the carbamate.

Experimental. Cats of either sex weighing between 1.5 and 3.1 kg were anesthetized with Dial-Urethane§ 0.66 ml/kg. The femoral trunk and the hamstring branches of the sciatic were cut. The central end of the cut tibial nerve freed for one cm from the branching of the sciatic trunk was stimulated with

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† Mephenesin and its ester were kindly supplied by W. A. Lott of The Squibb Institute of Medical Research.

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§ Kindly supplied to us by Dr. Yonkman of Ciba Pharmaceutical Co.

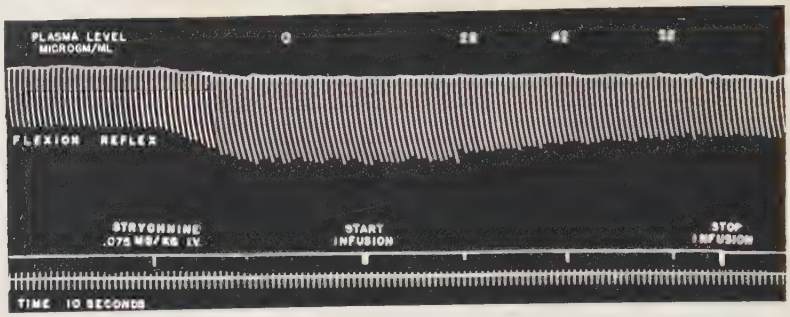

FIG. 1. Typical experiment showing effects of infusing 1.25 mg/kg/min. of mephenesin. Cat 2.0 kg. Stimulus 0.1 millisec, 12.5 v. Plasma level of 52 μg/ml should be at the time infusion was stopped (10 minutes).

shielded electrodes. Recordings were made on a smoked drum with a spring tension lever attached to the cut tendon of the tibialis anticus muscle. Stimuli were delivered at the minimum rate of the Grass Model S-4-A stimulator used (one per 9.5 sec.). The response to supramaximal stimulation as measured by excursion of the writing lever was used in all the experiments reported here. The voltage was greater than 2 x maximal, ranging from 12-25 volts at 0.1 msec. duration. The leg was fixed in position by using a clamp on the femur and another on the paw. Infusions and injections were made into the jugular vein cannulated with a long piece of polyethylene tubing reaching the Engineering Specialties Constant Infusion Machine Model 4C.‖ This pump allows reproducible rates from 0 to approximately 13 ml/min. with an accuracy of $\pm 1\%$. It was calibrated by timing repeatedly at various settings the delivery of 2 ml of saline into a Normax burette. Infusions were done at one ml/kg/min., varying concentrations of the drugs being used. Infusion rates ranged from 1.0-1.75 mg/kg/min. for the carbamate and from 1.25-1.65 mg/kg/min. for mephenesin. The dead space of the polyethylene tubing was corrected for by introducing a small air bubble into it and starting the timing of the infusion when this bubble reached the animal. There was no danger of accidentally changing the position of the animal during the experiment. The only time the experimentor came close to the animal was when the clamp on the carotid was removed to allow a blood sample to be taken.

After allowing a control period of 5-15 minutes, strychnine sulfate 0.075-0.10 mg/kg was injected. If the increase in excursion was unsuitable, which occurred with only very few of the animals, another ½ of this dose was administered. After allowing sufficient time for the strychninized animal to equilibrate, a control blood sample was taken and the infusion started. Blood samples were taken every 3 minutes thereafter until the infusion was terminated. Ten to 20 seconds were needed to collect the blood. Control experiments without infusion showed that the strychnine effect lasted for considerably longer than any infusion period reported herein.

Results. A typical record obtained by the method described is seen in Fig. 1. Seven animals were used with each of the drugs. The interpretation of the experiments was made as follows: 1. The mean of 10 heights of contraction was calculated for the control period, the control period after strychnine, and at the times blood samples were taken. 2. The increase in height of contraction over control after the injection of strychnine was arbitrarily equated to 100%. The reduction in twitch height could then be assigned a value of "increment in arbitrary per cent." 3. A plot of % increment (see 2) *vs.* plasma level of the compound was made for each experiment and a smooth curve was fitted. 4. Values of plasma levels at arbitrary 90%, 80%, 70%, etc. increment were read from this curve. 5. The means of values from No. 4 for all 7 experiments for each drug were determined, expressed in μg/ml, and converted to

‖ Obtained from Engineering Specialties, Madeira, Ohio.

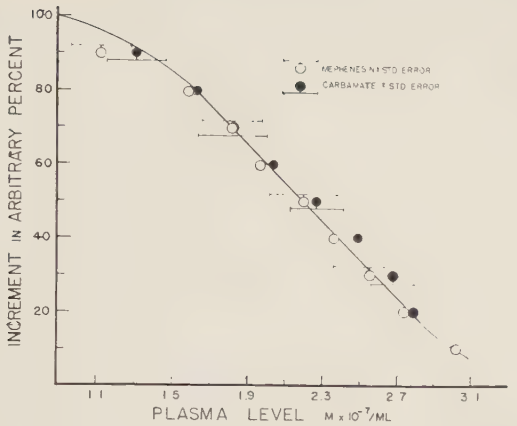

FIG. 2. Plot of mean plasma levels of mephenesin and its carbamate against increment in flexion reflex after strychnine (see text).

Moles x 10^{-7}/ml. These values were then plotted as in No. 3 to obtained the curve seen in Fig. 2.

Knowing that the plasma level ± its standard error at the 50% increment point for the carbamate is 2.27 ± 0.13 M x 10^{-7}/ml and that for mephenesin 2.20 ± 0.11 M x 10^{-7}/ml there is obviously no need for further statistical evaluation to see that the 2 compounds are equal in potency on a molar basis. On a microgram basis the carbamate is significantly less potent than the parent compound.

Discussion. These results cannot be interpreted as indicating that the carbamate is converted to mephenesin in order to become active since it has been shown by several observers(5,6) that there is no significant excretion of beta-toloxy lactic acid, the main metabolic product of mephenesin, after the administration of large doses of mephenesin carbamate. Richardson(4,8) found the ester to be excreted mainly in the free and in a conjugated form (probably with glucuronic acid). We are dealing here, therefore, with a derivative which in itself has the action of mephenesin, with equal potency according to this technic. It has been shown(4) that it requires the infusion of less carbamate than of mephenesin after a large priming dose to maintain a constant plasma level.

We must conclude, therefore, that any difference in action on multineuron reflexes, *i.e.*, duration after a constant molar dose, is due to differences in physiologic disposition of the compounds. The rapid metabolism of mephenesin must be considered one of the most important factors in any such difference.

Changes in the rate of infusion in these experiments unfortunately tell very little about the comparative rates of distribution and disposition of the 2 drugs. A very great variability was encountered, with both compounds, in the time course of buildup of plasma level. This variability was perhaps somewhat greater with mephenesin itself. The analytical method detected only non-conjugated, unmetabolized mephenesin and mephenesin carbamate. The possibility of a difference in potency at the receptor site is not completely ruled out. It has been shown in dogs, however, that the tissue plasma ratio in brain at high plasma levels is approximately the same for both compounds(4,5).

Summary. Mephenesin and its ester with carbamic acid have been shown to be of equal potency when their effect on a multineuron reflex was correlated with their plasma levels.

We would like to thank Dr. I. H. Slater for a most helpful discussion of the problem with one of us (PED).

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Abnormal Tryptophan Metabolites in Human Pregnancy and their Relation To Deranged Vitamin B_6 Metabolism. (21183)

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After ingestion of 10 g of Dl-tryptophan, normal individuals excrete only traces of xanthurenic acid (4-8 dihydroxquinoline-2-carboxylic acid). However, in subjects with induced vit. B_6 deficiency the same tryptophan load test results in the excretion of appreciable amounts of this metabolite in the urine(1). A similar disturbance of tryptophan metabolism occurs in pregnancy but can be promptly corrected by the administration of pyridoxine (2,3). Paper chromatographic methods have recently disclosed that after feeding tryptophan, additional metabolites are excreted by vit. B_6 deficient, but not by normal rats(4). We wish to report a similar tryptophan metabolite excretion pattern in normal pregnant women, which is readily corrected by the administration of pyridoxine.

Material and methods. Ten gram doses of Dl-tryptophan were given to 4 normal controls, and to 11 women one to 2 days after delivery. The urine was collected for 24 hours and xanthurenic acid determined quantitatively, as previously described(5). The excretion of the normal controls averaged 16 mg and that of women at term 183 mg of xanthurenic acid. To isolate the tryptophan metabolites, the method of Dalgliesh(4) was used. All urines were treated identically to enable semi-quantitative correlation. Mercuric acetate in slight excess was added to 2/10 of the acidified 24-hour specimen. The formed precipitate was treated with H_2S. Excess H_2S was then expelled from the supernatant containing the released metabolites with the help of nitrogen. Deactivated charcoal was then added to absorb completely all fluorescent compounds. The charcoal was eluted to remove the fluorescent substances—generally 9 washings of 5% phenol were sufficient—and the combined yellow eluates were vacuum concentrated to 2 ml.

Four λ aliquots were chromatographed on Whatman paper No. 1 using the descending technic with a solvent mixture of butanol, acetic acid and water (4:1:5). Available reference substances included L-kynurenine, Dl-kynurenine, 3-hydroxy-knurenine, xanthurenic acid, kynurenic acid and Nα-acetyl-kynurenine. Spots appearing under fluorescent light are recorded in Table I as well as R_f values, results of various color tests and correlation to the spots described for vit. B_6 deficient rats(4). Concentrated urine preparations were also hydrolyzed in a sealed tube with 6 N H_2SO_4 for one hour at 100°C and chromatographed after removal of the acid.

Results. Three spots, No. 6, 7, and 8 were always found in the controls and occasionally a trace of No. 10. In pregnancy urine these spots exhibited stronger fluorescence and at least 6 additional spots numbers 1, 2, 3, 4, 5, and 9 were recognized. Spot 10 was always present. After acid hydrolysis only spots 5, 6, and 8 remained.

Reference to Table I indicates that spots 5 and 6 can be clearly identified as 3-hydroxy-kynurenine and kynurenine, respectively. Available reference standards enabled direct comparison and proof. Spot 6 was composed of 2 distinct parts, the lower part, representing the D-isomer, formed a narrow band on the leading edge of 3-hydroxy-kynurenine. This indicates some conversion of D-tryptophan to d-kynurenine. If only L-tryptophan was given the D-isomer was absent. In pregnancy urines, spot 8 was apparently a mixture of kynurenic and xanthurenic acids, and gave a positive Pauly reaction. In contrast, spot 8 in normal subjects gave a negative reaction, thus confirming the absence of an appreciable amount of xanthurenic acid, as previously established by chemical estimation. Therefore, spot 8 in non-pregnant subjects is probably kynurenic acid only. Spot 9 is identical with spot 1 found by Dalgliesh in vit. B_6 deficient rats and seems to be a conjugated 3-hydroxy-kynurenine. Spot 10, compared with Nα-acetyl-kynurenine as reference material gave identical reactions. Spots 1, 2,

TABLE I. Summary of Experimental Data.

Spot #	Mean Rf	Fluorescence	Color reactions					Reference substance available	Correlation with Dalgliesh spots(4)	Probable identity	Occurrence in	
			Ninhydrin	Ehrlich	Ekman	Pauly	Ammoniacal silver nitrate				Controls	Pregnancy following 10 g dl-tryptophan
1	14	Weak bluish-green	Red-purple	Faint pink	—	—	—	No	A?	Deriv. of 3-hydroxy-kynurenine?	—	+
2	18	Strong bluish-green	Purple	Pink	Yellow, on diazotising turning brown	Red-brown	Brown	"	C?	*Idem*	—	+
3	21	Brilliant blue-purple	—	—	—	—	—	"			—	+
4	24	Brilliant blue-purple	—	—	—	Red	—	"		Derivative of xanthurenic acid?	—	+
5	41	Green	Brown-purple	Pink	Yellow, on diazotising turning brown	Red-brown	Brown-black	Yes	E	3-hydroxy-kynurenine	—	+
6	49	Strong pale blue	Purple	Orange	Magenta	—	Brown	"	F	Kynurenine	+	+
7	55	Deep blue-purple	—	—	—	—	—	No			+	+
8	63	Blue-purple	—	—	—	Red	Faint brown	Yes	H	Kynurenic and xanthurenic acid	+*	+
9	83	Green	—	Pink	Yellow, on diazotising turning brown	Red-brown	Brown-black	No	I	N α-acetyl-3-hydroxy-kynurenine	—	+
10	86	Pale blue	—	Orange	Magenta	—	Brown	Yes	J	N α-acetyl-kynurenine	±	+

* Apparently kynurenic acid only.

5, and 9 appear yellow on the untreated chromatogram. On the basis of its color reactions, fluorescence and disappearance on hydrolysis, spot 2 is apparently a derivative of 3-hydroxy-kynurenine. Spot 1 may also be a derivative of 3-hydroxy-kynurenine. Its inconsistent color reactions are probably due to its low concentration. Spots 3 and 4 showed a brilliant blue-purple fluorescence and the strong reaction with Pauly's reagent given by spot 4 would suggest that it is a derivative of xanthurenic acid. Spot 7 had a deep purple fluorescence and gave no typical color reactions.

In 3 women at term the tryptophan load test was repeated after an interval of 24 hours. During this time 50 mg of pyridoxine HCl were given in 2 equal doses 12 hours and ½ hour before the administration of the second dose of 10 g of Dl-tryptophan. In agreement with previous findings, no xanthurenic acid could be detected in the urine after administration of the vitamin(3). Chromatographic analyses of these urines showed complete disappearance of spots 5 and 9 and the merest traces of fluorescence in spots 1, 2, 3, 4, and 10. The intensity of spots 6, 7, and 8 resembled those obtained in non-pregnant normal controls.

Comment. The excretion of various tryptophan metabolites in subjects on a regular hospital diet even without added tryptophan has been reported. Thus, children with congenital hypoplastic anemia excrete large amounts of anthranilic acid(6). Spaček described the increased excretion of kynurenine and of another unidentified substance in conditions associated with cachexia such as cancer(7). In the urine of a patient with severe tuberculosis, Makino and his coworkers demonstrated the presence of kynurenine, 3-hydroxy-kynurenine, kynurenic and xanthurenic acids and conjugation products of these substances(8). Kotaki and Tani found xanthurenic acid and 3-hydroxy-kynurenine in the urine of patients with diabetes(9). Dalgliesh and Tekman investigated a large number of patients with various diseases and found excretion of 3-hydroxy-kynurenine only in patients with febrile states regardless of their etiology(10). These investigators attributed the excretion of abnormal tryptophan metabolites to a high rate of breakdown of body proteins causing a relative insufficiency of the enzyme systems responsible for tryptophan catabolism.

Following the ingestion of 10 g Dl-tryptophan, we found several fluorescent spots in the chromatograms prepared from the urines of our normal controls. Three of these spots could be identified as kynurenine, kynurenic acid and Nα-acetyl-kynurenine, respectively. Dalgliesh and Tekman detected only kynurenine in the urine of 2 control subjects under the same conditions. The finding of kynurenic acid and acetylated kynurenine is, however, of some interest, since besides being attacked by kynureninase, kynurenine formed can be removed in 3 other ways: a) by oxidative deamination or transamination to give, after ring closure, kynurenic acid, b) by conjugation, and c) by ring oxidation to give hydroxy-kynurenine(10).

In pregnancy many more tryptophan metabolites appear in the urine in an excretion pattern strikingly similar to that observed in the vit. B_6 deficient rat. It is of interest to note that abnormal tryptophan metabolites in induced vit. B_6 deficiency occur not only in the rat but also in the rice moth larva(11).

The ability of vit. B_6 to rectify this abnormal excretion pattern strongly suggests that in pregnancy there exists a vit. B_6 deficiency which is probably conditioned by the increased demand of the growing fetus for this essential vitamin(12).

Summary. Following ingestion of 10 g Dl-tryptophan, normal subjects excrete kynurenine, kynurenic acid and occasionally Nα-acetyl kynurenine. In pregnancy many additional metabolites, including 3-hydroxy-kynurenine, occur in the urine. The abnormal tryptophan metabolite excretion pattern in these women is strikingly similar to the one described in vit. B_6 deficient rats. Administration of pyridoxine suppresses to a large degree the excretion of the abnormal substances.

We should like to thank Professor A. Butenandt for a sample of 3-hydroxy-kynurenine and Dr. D. M. Bonner for samples of l-kynurenine and Nα-acetyl-kynurenine. Dl-Tryptophan and Pyridoxine hydro-

chloride were obtained through the courtesy of Dr. E. Alpert from Merck and Co., Rahway, N. J.

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Preparation of Globin from Human Hemoglobin. (21184)

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Considerable interest has been shown in the preparation of plasma expanders from human globin(2,4,5). For this purpose it is not necessary to utilize so-called "undenatured" globin, but it is desirable to use a colorless globin protein which can then be modified as desired. The usual methods of preparing globin protein from hemoglobin are those of Anson and Mirsky(1), Strumia and Chornock(2) and Strumia and Sample(3). These methods utilize the precipitation of a globin hydrochloride from a reaction mixture obtained by mixing an aqueous hemoglobin solution with acidified acetone. The globin obtained by these procedures is unfortunately contaminated with considerable quantities of heme or heme complexes. The purpose of this communication is to describe a new method of preparing a globin protein which is almost devoid of colored impurities.

This new procedure is based on two findings: 1, a true solution of dissociated human hemoglobin can be obtained in certain mixtures of water, water-miscible solvents and strong acids; and 2, activated carbon will adsorb the heme from such solutions. Thus, the method consists of dissolving hemoglobin in a suitable acid-water-solvent solution, treating the solution with activated carbon, filtering off the carbon, and recovering the globin from the filtrate. One suitable set of conditions is described below.

Materials and methods. Fresh, packed human red blood cells at 10°C were filtered through cheese cloth to remove clots. The filtered cells were lysed by the addition of 2 volumes of distilled water at room temperature and the solution adjusted to pH 5.8 ± 0.1 with 1 N HCl. The precipitated stroma was removed by centrifugation. To 100 ml of stroma-free lysate, 300 ml of acetone containing 5 ml of 37% hydrochloric acid were added slowly with stirring. As soon as a clear solution was obtained, 15 g of Darco G-60* were added and the mixture stirred gently for 10 minutes. Seventy-five ml of distilled water were added to the slurry which was stirred for an additional 20 minutes. The slurry was then filtered with vacuum through a Buchner funnel. One gram of Hyflo Super Cell† was added to the carbon slurry to speed filtration.

The filtrate was adjusted to pH 7.0 with 5% sodium hydroxide. One hundred and fifty ml of acetone were added with stirring to precipitate the globin which was then collected by filtration. The globin can also be obtained

* Obtained from Darco Department, Atlas Powder Co.

† Obtained from Johns Mansville.

in the acid form by precipitation from the Darco G-60 filtrate by the addition of 500 ml of acetone. The globin, or acid globin, was dried by slurrying twice in 150 ml volumes of dry acetone and air-drying after each suspending operation.

Results and discussion. *Sixty-five to 70%* of the nitrogen in the original lysate was obtained in the globin. $E^{1\%}_{1\,cm}$ at 403 mμ was 0.032 for an aqueous solution of acid globin prepared by the method described. This is compared to a value of 0.134 for a solution of acid globin prepared by the method of Strumia and Chornock(2). The solubilities of the 2 products are similar.

Many modifications of the above procedure can be made. Other solvents such as ethanol, dioxane, and mixtures of acetone and ether or acetone and methyl ethyl ketone have been used with varying degrees of success. The addition of water to the carbon slurry may be eliminated with a slight decrease in globin recovery. The efficiency of heme removal increases with the hydrochloric acid concentration up to 0.25 N, with a concurrent decrease in globin recovery. Hydrochloric acid was the most efficient acid tested; sulfuric acid and citric acid produced only fair heme removal. The concentration and type of activated carbon have a large effect on the quality of the globin produced. Concentrations of Darco G-60 greater than 15% produced globin with still less color, but the yields were considerably smaller. Darco G-60 was the most efficient of 11 brands of activated carbon tested.

The temperature at which the adsorption is carried out (from 0° to 30°C) has only a slight effect on the color quality of the globin produced. The yield of globin is less at lower temperatures (0° to 10°C), probably due to the decreased solubility of the globin at the lower temperatures.

Care must be exercised during the process to keep down oxidative reactions. Such precautions as the use of fresh red cells and only very gentle stirring during all mixing operations are essential if a product low in color is to be obtained.

Summary. A method is described of preparing nearly colorless globin protein from human hemoglobin. The method consists of adsorbing the heme on carbon from acidic solution of dissociated hemoglobin and then precipitating the globin protein from the solution after filtration to remove the carbon.

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